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N-Acylethanolamines: Formation and Molecular Composition of a New Class of Plant Lipids[1]

Kent D. Chapman*, Swati Tripathy, Barney Venables, and Arland D. Desouza

Department of Biological Sciences, Division of Biochemistry and Molecular Biology, University of North Texas, Denton, Texas 76203–5220 (K.D.C., S.T., A.D.D.); and TRAC Laboratories, 113 South Cedar, Denton, Texas 76201 (B.V.)

Recently, the biosynthesis of an unusual membrane phospholipid, *N*-acylphosphatidylethanolamine (NAPE), was found to increase in elicitor-treated tobacco (*Nicotiana tabacum* L.) cells (K.D. Chapman, A. Conyers-Hackson, R.A. Moreau, S. Tripathy [1995] Physiol Plant 95: 120–126). Here we report that before induction of NAPE biosynthesis, *N*-acylethanolamine (NAE) is released from NAPE in cultured tobacco cells 10 min after treatment with the fungal elicitor xylanase. In radiolabeling experiments [^{14}C]NAE (labeled on the ethanolamine carbons) increased approximately 6-fold in the culture medium, whereas [^{14}C]NAPE associated with cells decreased approximately 5-fold. Two predominant NAE molecular species, *N*-lauroylethanolamine and *N*-myristoylethanolamine, were specifically identified by gas chromatography-mass spectrometry in lipids extracted from culture medium, and both increased in concentration after elicitor treatment. NAEs were found to accumulate extracellularly only. A microsomal phospholipase D activity was discovered that formed NAE from NAPE; its activity in vitro was stimulated about 20-fold by mastoparan, suggesting that NAPE hydrolysis is highly regulated, perhaps by G-proteins. Furthermore, an NAE amidohydrolase activity that catalyzed the hydrolysis of NAE in vitro was detected in homogenates of tobacco cells. Collectively, these results characterize structurally a new class of plant lipids and identify the enzymatic machinery involved in its formation and inactivation in elicitor-treated tobacco cells. Recent evidence indicating a signaling role for NAPE metabolism in mammalian cells (H.H.O. Schmid, P.C. Schmid, V. Natarajan [1996] Chem Phys Lipids 80: 133–142) raises the possibility that a similar mechanism may operate in plant cells.

NAPE is a widespread, albeit minor, membrane phospholipid in animal and plant tissues (Schmid et al., 1990; Chapman and Moore, 1993). Its unusual structural features (a third fatty acid moiety linked to the amino head group of PE) impart stabilizing properties to membrane bilayers (Domingo et al., 1994; LaFrance et al., 1997). NAPE and its hydrolysis products, NAEs, are known to accumulate in vertebrate tissues under pathological conditions (for review, see Schmid et al., 1990). Recently, there has been renewed interest in NAEs because of the contention that anandamide (*N*-arachidonylethanolamine) is an endogenous ligand for the cannabinoid receptor in mammalian brain (Devane et al., 1992; Fontana et al., 1995; Schmid et al., 1996). The likely route for NAE formation in neural and nonneural tissues, although the matter of some debate, is via the signal-mediated hydrolysis of NAPE (DiMarzo et al., 1994; Schmid et al., 1996; Sugiura, et al., 1996).

In plants little is known regarding the catabolism of NAPE. In cottonseed microsomes NAPE was metabolized to NAE or NAlysoPE by PLD- or PLA-type activities, respectively (Chapman et al., 1995b). However, the metabolic fate of NAPE in vivo and the factors that regulate NAPE hydrolysis remain largely unknown. We previously noted that the biosynthesis of NAPE was increased in elicitor-treated cell suspensions of tobacco (*Nicotiana tabacum* L.). Here we extend our investigations with this model system to examine NAPE catabolism by plant cells in vivo. NAE was released from NAPE, and it accumulated extracellularly. We identified by GC-MS these tobacco NAEs as *N*-lauroylethanolamine and *N*-myristoylethanolamine. These NAEs were increased in elicitor-treated cell suspensions. Furthermore, we detected the enzymatic machinery capable of the release and the degradation of NAEs in tobacco cells. To our knowledge this represents the first identification of the NAE molecular species in plant cells. It is tempting to speculate that NAPE hydrolysis in elicitor-treated plant cells may be involved in a signaling pathway analogous to that found in mammalian cells.

MATERIALS AND METHODS

Cell Cultures, Elicitor Treatment, and Lipid Extractions

Tobacco (*Nicotiana tabacum* L. cv KY-14) cell suspensions were subcultured every 7 d (Chapman et al., 1995a); cell suspensions in log phase were treated with elicitor (xylanase, 1 μg/mL) as previously described (Chapman et al., 1995a). Control and experimental treatments were carried out on aliquots of the same population of cells. Culture supernatants were separated from cells by filtration. Cells were quick frozen in liquid N_2, powdered in a mortar, and

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* Corresponding author; e-mail kent@jove.acs.unt.edu; fax 1-940-565-4136.

Abbreviations: EIMS, electron-impact mass spectrum(a); NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PE, phosphatidylethanolamine; PLA, phospholipase A; PLD, phospholipase D; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

added to boiling 2-propanol in a ratio of 0.8:2 (grams fresh weight of cells:milliliters of 2-propanol). Culture supernatants were added directly to 2-propanol in the same ratio (v/v) without freezing. Lipids were extracted from samples with chloroform (Bligh and Dyer, 1959). For radiolabeling experiments in vivo, cells in log phase (3–4 d after subculture) were incubated for 4 h with [1,2-^{14}C]ethanolamine (2 μCi; 3 μCi μmol^{-1}, NEN) before treatment with elicitor.

Lipid Analyses

To assess radiolabeled ethanolamine-containing lipids, total lipids were subjected to TLC and radiometric scanning, as described previously (Chapman and Moore, 1993; Chapman et al., 1995a, 1995b). For structural characterization, NAE was separated from the total lipid extracts by a combination of Si gel cartridge chromatography and TLC (Chapman and Moore, 1993; Chapman et al., 1995b). Ethanolamine-containing lipids were identified on TLC plates by co-chromatography with authentic standards (Chapman and Moore, 1993; Chapman et al., 1995b; Sandoval et al., 1995). NAE-enriched fractions were recovered from Si gel plates in chloroform, *O*-acetylated (Fontana et al., 1995), and analyzed by GC-MS (model 5970 mass spectrometer equipped with a capillary interface to a model 5890 series II gas chromatograph, Hewlett-Packard). Derivatized samples in chloroform were chromatographed on a 30-m × 0.25-mm capillary column (Supelcowax 10, Supelco, Bellefonte, PA) with an oven temperature program of 100°C for 2 min, increased to 240°C at 10°C/min, and then held at 240°C for an additional 32 min. The injector temperature was 200°C and the inlet carrier gas (He) was 5 p.s.i. Synthetic NAEs were treated in the same manner, but with different TLC plates and glassware as a precaution to avoid contamination with these analytes. Synthetic NAE molecular species were kindly provided by Dr. Daniele Piomelli (The Neurosciences Institute, San Diego, CA) and their purity was verified by GC-MS.

Tobacco cell NAPE was purified by TLC and digested with *Streptomyces chromofuscus* PLD (Chapman and Moore, 1993). The NAEs derived from NAPE were derivatized and analyzed by GC-MS (as described above).

PLD and Amidohydrolase Assays

Tobacco cells (approximately 12 g fresh weight) were homogenized and microsomes isolated as previously described (Chapman et al., 1995a, 1995b). Radiolabeled NAPE was prepared fresh for each experiment from equimolar amounts of *sn*-1,2-dioleoylphosphatidyl[2-^{14}C]ethanolamine (31.5 nmol; 31.8 nCi/nmol, Amersham) and palmitoyl chloride (Dawson et al., 1969) and purified by one-dimensional TLC (Chapman and Moore, 1993; Chapman et al., 1995b). Approximately 20,000 dpm were used per assay, and samples were sonicated briefly after the addition of the substrate (in 20 μL of diethyl ether). Reactions were carried out at 32°C in a final volume of 1 mL with shaking (120 rpm). Assays were buffered with potassium phosphate (100 mM, pH 6.0) and were started by the addition of the substrate, ^{14}C-labeled NAPE. Assay reaction mixtures were incubated for 30 min and stopped by the addition of 2 mL of boiling 2-propanol. Lipids were extracted from the alcohol-water mixture into chloroform and separated by TLC (Chapman et al., 1995b). Released NAE was quantified by radiometric scanning (Bioscan System 200 Imaging Scanner, Bioscan, Washington, DC) and/or liquid-scintillation counting as described previously (Chapman et al., 1995b).

NAE amidohydrolase activity was measured by following the hydrolysis of [^{14}C]NAE (release of water-soluble [^{14}C]ethanolamine). [^{14}C]NAE was prepared by enzymatic digestion of [^{14}C]NAPE (prepared as described above) with *S. chromofuscus* PLD (Chapman and Moore, 1993). Approximately 5000 dpm of [^{14}C]NAE (in a small volume of methanol) was added to aliquots of homogenates or microsomes and incubated for 30 min at 30°C. Lipids were extracted from enzyme reaction mixtures, and radioactivity in aqueous and organic fractions was quantified by liquid-scintillation counting. Enzyme activity was calculated based on the radiospecific activity of the original [^{14}C]dioleoyl PE used for NAPE synthesis (as described above).

RESULTS

Radiolabeling experiments in vivo (ethanolamine-containing lipids were specifically radiolabeled with [1,2-^{14}C]ethanolamine) demonstrated the occurrence of a xylanase-stimulated NAE release into the culture medium 10 min after treatment (Table I). This release appeared to be at the expense of NAPE, because radiolabeled cellular NAPE declined dramatically in elicitor-treated cells. There was little relative change in other ethanolamine-containing lipids. Replicate experiments, although varying in the efficiency of incorporation of radiolabel into lipids, consistently showed a release of NAE at the expense of NAPE when cells were treated with elicitor. The decrease in radiolabeled NAPE was not completely accounted for by the increase in radiolabeled NAE in the culture medium. We

Table I. *Release of ^{14}C-labeled NAE from elicitor (xylanase)-treated tobacco cells (5 g fresh weight)*

Values represent the mean dpm and SD of four independent experiments.

Cell	Medium	Cell
	dpm/flask[a]	
Control (no xylanase)		
Lipid class		
NAE	506 ± 54	649 ± 95
NAPE	–	8,651 ± 2,462
PE	–	154,396 ± 25,112
Other (mostly phosphatidylcholine)	–	15,921 ± 2,528
Xylanase-treated (10 min)		
Lipid class		
NAE	3104 ± 920	449 ± 131
NAPE	–	1,593 ± 382
PE	–	155,432 ± 56,207
Other	–	16,646 ± 1,852

[a] Radiolabeled for 4 h with [1,2-^{14}C]ethanolamine.

speculate that an amidohydrolase activity (see below) may be responsible for the subsequent metabolism of NAE.

The NAEs that were specifically released into the tobacco cell culture medium after xylanase treatment were identified by GC-MS as *N*-lauroylethanolamine (NAE 12:0) and *N*-myristoylethanolamine (NAE 14:0) (Fig. 1). EIMS and retention times in GC of the *O*-acetylated derivatives of the endogenously released NAEs from tobacco cells were identical with authentic standards. Other types of NAEs (longer or unsaturated acyl chains) were not detected in tobacco cell suspensions. There was a measurable increase in both NAE molecular species 10 min after xylanase treatment, although not as pronounced as that inferred from radiolabeling experiments. In GC-MS experiments NAE 12:0 and NAE 14:0 in the culture medium were estimated to increase about 2-fold after elicitor treatment (from 5.6 to 10.0 and 3.6 to 8.4 ng/g fresh weight of cells, respectively). The apparent discrepancy between results from radiolabeling experiments and results from GC-MS experiments may be attributable to losses during processing/derivatization of samples for GC.

Single-ion chromatograms (*m/z* 145, major diagnostic ion characteristic of NAEs) for NAE-enriched samples from cells and culture medium of unelicited and elicitor-treated cells are compared in Figure 2. NAE 12:0 and NAE 14:0 (identified by their respective EIMS, Fig. 1) were detected in culture medium, but were barely detectable in lipids from cells. These data suggest that NAEs are released into the extracellular medium and do not accumulate intracellularly. The low levels of intracellular NAEs may be caused by the degradation of these molecules by an amidohydrolase activity (see below). In separate experiments we confirmed that lauric acid and myristic acid were present endogenously as the predominant *N*-acyl constituents of tobacco NAPE (not shown). Longer *N*-acyl chains, as previously identified in cottonseed (Chapman and Moore, 1993; Sandoval et al., 1995), were not detected in tobacco cells. This may be indicative of different physiological roles for NAPE metabolism in these different cell types or different developmental stages (germinated seeds versus cell suspensions).

Two other abundant lipid species in these chromatograms, one at 48.5 min and one at 59.8 min, were present in the cell and medium samples, but appeared more abundant in the medium after elicitor treatment (similar to the results for NAEs). EIMS of these species (not shown) indicated that they were likely bis-*O*-acetylated derivatives of monoacylglycerols, palmitylglycerol and stearylglycerol, respectively. Diagnostic ions in both spectra of $[M\text{-}175]^+$, representing the fatty acyl moiety, were evident (at *m/z* 239 for palmityl and at *m/z* 267 for stearyl). Also, the prominent ion in both spectra was at *m/z* 159, which would correspond to the derivatized glyceryl fragment ion. Although our experimental results do not address the origin of these lipids, their occurrence suggests the involvement of PLA (and phospholipase C, or PLD and phosphatidic acid phosphatase) action on membrane phospholipids.

A microsomal enzyme activity from tobacco cells was identified that hydrolyzed NAPE to NAE in vitro (Fig. 3). The tobacco PLD-type activity was present in cytosolic fractions as well. NAE formation by tobacco microsomes was stimulated somewhat by Ca^{2+} and GTP-γ-S. However, the activity was inhibited by concentrations of Triton X-100 that are known to stimulate a similar PLD activity previously characterized in mammalian liver (Schmid et al., 1996). Most notably, the tobacco microsomal PLD activity toward exogenously supplied [^{14}C]NAPE was increased about 20-fold in the presence of mastoparan, suggesting the possibility of G-protein-mediated regulation of NAPE hydrolysis. It should be pointed out that a direct stimulation of tobacco PLD activity by mastoparan, as opposed to involvement of an activated G-protein, cannot be ruled out. The xylanase protein preparation itself had no hydrolytic activity toward NAPE in vitro, and adding elicitor to the microsomes did not stimulate PLD activity above control levels (not shown).

Because only trace levels of NAEs were detected in cellular lipid extracts, we postulated that there was an intracellular NAE amidohydrolase activity like that found in some mammalian tissues (Desarnaud et al., 1995; Ueda et al., 1995) that could catalyze the hydrolysis of NAE. Such an activity was readily detected in tobacco cell homoge-

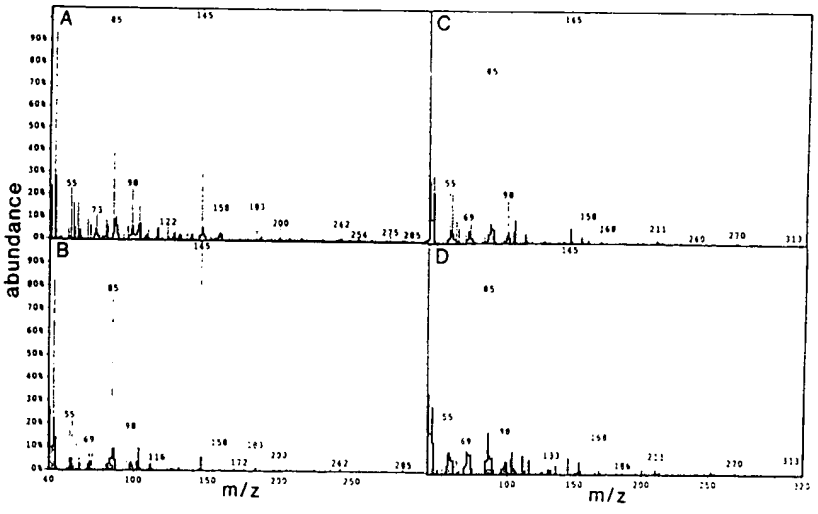

Figure 1. EIMS of NAEs. *O*-Acetylated derivatives of putative tobacco NAE 12:0 (A), synthetic NAE 12:0 (B), synthetic NAE 14:0 (C), and putative tobacco NAE 14:0 (D) were analyzed by GC-MS. Retention times in GC and molecular ions $[M]^+$ in EIMS of tobacco NAEs were identical to those of synthetic compounds (33.5 min and *m/z* 285 for NAE 12:0; 45.6 min and *m/z* 313 for NAE 14:0). Before GC-MS, tobacco NAEs were partially purified by TLC from total lipid extracts and derivatized according to Fontana et al. (1995). The synthetic NAEs were treated in the same manner, but with different TLC plates and glassware as a precaution to avoid contamination.

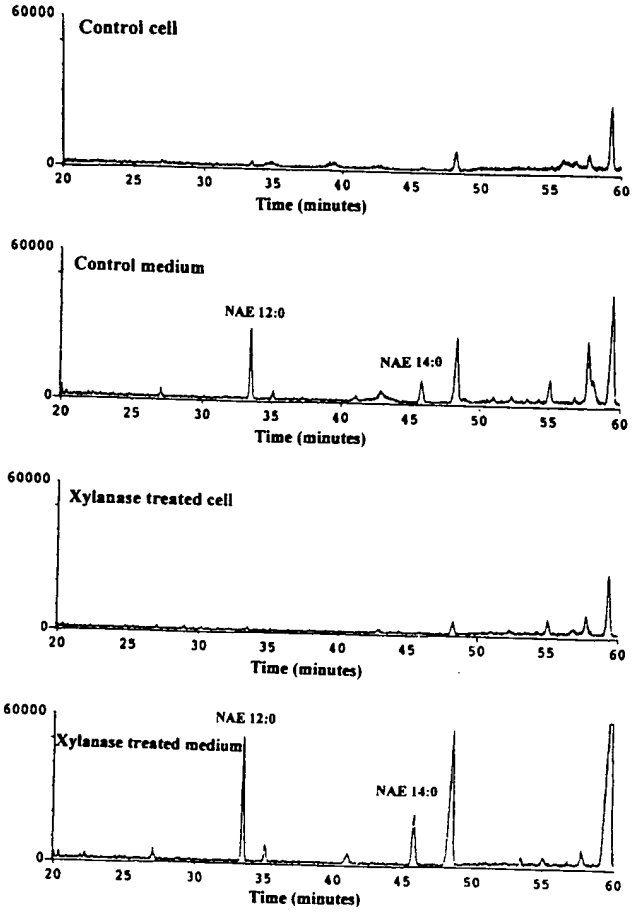

Figure 2. Single-ion chromatograms at *m/z* 145 of *O*-acetylated, NAE-enriched samples from unelicited (control) cells and medium and xylanase-treated cells and medium. Peaks identified by electron-impact MS as NAE 12:0 and NAE 14:0 are labeled. Other lipid molecules in the chromatograms did not show mass spectra characteristic of NAEs (Fontana et al., 1995; see text).

nates and membranes (Table II), although the latter accounted for only a small proportion of the total activity. This hydrolytic activity released water-soluble radioactivity from [^{14}C]NAE (radiolabeled on carbon 2 of the ethanolamine) and was inactivated by boiling the cell fractions. Consequently, we conclude that an amidohydrolase-type activity is present in tobacco cells and could be responsible for the rapid removal of free intracellular NAE.

DISCUSSION

Recent studies indicated that NAPE biosynthesis was increased in elicitor-treated tobacco cells (Chapman et al., 1995a). Approximately 2 h were required for the maximum induction of NAPE biosynthesis, as judged by enzyme activity and lipid accumulation, suggesting that NAPE biosynthesis was not involved in the early membrane permeability-related events of pathogen perception. Instead, we hypothesized that the increase in NAPE biosynthesis might be required to replenish NAPE levels depleted by the signal-mediated hydrolysis of NAPE. The results presented here are consistent with such a hypothesis.

The tobacco-cell/fungal-elicitor model system has been used by a number of research groups to identify and characterize various components involved in plant defense responses. These include changes in ion flux across the plasma membrane (Bailey et al., 1992), changes in plasma membrane lipid metabolism (Moreau and Presig, 1993; Moreau et al., 1994; Chapman et al., 1995a), transient protein (Tyr) phosphorylation (Suzuki and Shinshi, 1995), induction of phytoalexin biosynthesis (Moreau and Presig, 1993), induction of ethylene biosynthesis (Anderson et al., 1993), induction of pathogenesis-related protein expression (Lotan and Fluhr, 1990), and induction of defense gene expression (Bailey et al., 1995; Suzuki et al., 1995). Recently, a receptor for the xylanase protein was identified in tobacco plasma membranes (Hanania and Avni, 1997), and a single gene trait has been linked to xylanase sensitivity (Bailey et al., 1993). Hence, our work on the regulation of NAPE metabolism in tobacco cells treated with xylanase

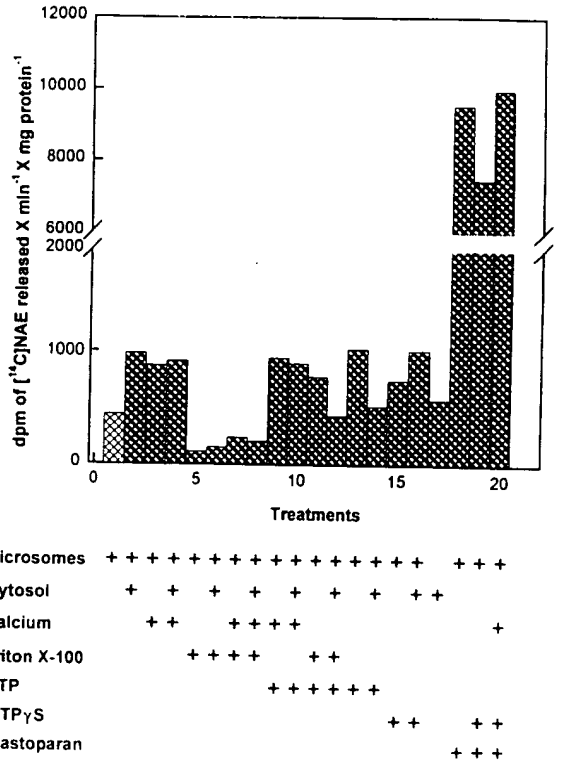

Figure 3. NAE formation from NAPE by tobacco microsomal PLD. Plus signs below the treatment indicate the addition of that component to the reaction mixture (microsomes, 0.04 mg of protein; cytosol, 0.025 mg of protein; calcium, 15 mM; Triton X-100, 2 mg/mL; GTP, 3 mM; GTP-γ-S, a nonhydrolyzable GTP analog, 25 μM; and mastoparan, 25 μM). Reactions were carried out at 30°C in a final volume of 1 mL with shaking (120 rpm). Assays were started by adding the substrate ([^{14}C-labeled NAPE) and stopped by adding 2-propanol. Released NAE was quantified by radiometric scanning (System 200 Imaging Scanner, Bioscan) (Chapman et al., 1995a) of TLC separations of lipids extracted from reaction mixtures. Values are the averages of duplicate samples (in all cases the range was less than 14%) and are representative of several replicate experiments.

Table II. *Enzymatic hydrolysis of N-palmitoyl[2-^{14}C]ethanolamine by homogenates and membranes of tobacco cells*

Activity was quantified as the amount of water-soluble radioactive [2-^{14}C]ethanolamine released after 30 min and is attributed to an amidohydrolase-type enzyme. Values represent the average enzyme activity and SD from three independent cell-fractionation experiments.

Cell Fraction	Total Activity	Specific Activity
	pmol h^{-1}	*units mg^{-1} protein*
Homogenate (650*g* supernatant)	1926 ± 465	66.3 ± 13.8
Membranes (150,000*g* pellet, 60 min)	121.0 ± 17.5	75.8 ± 6.9

may be relevant to signal-transduction pathways in plant defense responses. Additional work to characterize the biological effects of NAEs on plant cells will be necessary to understand the possible role of NAEs in plant cell signaling.

Other workers have implicated PLD induction in plant pathogen perception or wounding (Ryu and Wang, 1996; Young et al., 1996; Wang, 1997). Young et al. (1996) reported changes in plasma membrane distribution of a rice PLD in response to bacterial pathogens. In other studies a mastoparan-stimulated PLD activity was reported in carnation petals and *Chlamydomonas eugametos* cells; however, the endogenous lipid substrate was not identified (Munnik et al., 1995). Our results are consistent with an emerging role for a highly regulated PLD activity(ies) (Causier and Milner, 1996; Ryu and Wang, 1996; Pappan et al., 1997; Wang, 1997) that is involved in signal transduction pathways in plants. Moreover, our studies identify at least one type of endogenous membrane lipid substrate for PLD and characterize for the first time to our knowledge the structure of the hydrolysis products, NAEs.

The NAEs identified here have shorter acyl chains than the biologically active neurotransmitters (anandamide; Devane et al., 1992) or the closely related sleep-inducing compounds (oleoylamide; Cravatt et al., 1995) found in mammalian brain. Nonetheless, there are many similarities in NAE metabolism evident from our results that are shared between plants and animals. First, the molecular origin of NAE appears to be a relatively minor membrane phospholipid (NAPE). Second, NAEs are released from NAPE in a signal-mediated fashion and accumulate extracellularly. A PLD appears to catalyze the formation of NAE and an amidohydrolase appears to be responsible for its intracellular degradation. Whereas a signaling role for these NAEs in plants has yet to be firmly established, the emerging role for NAE as a signaling molecule in mammalian tissues (Schmid et al., 1996) and the similarities in its metabolism suggest that this mechanism may be widespread in evolution.

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N-Acylethanolamines in Seeds. Quantification of Molecular Species and Their Degradation upon Imbibition[1]

Kent D. Chapman*, Barney Venables, Robert Markovic, Raymond W. Blair, Jr., and Chris Bettinger

Department of Biological Sciences, Division of Biochemistry and Molecular Biology, University of North Texas, Denton, Texas 76203–5220 (K.D.C., R.M., R.W.B., C.B.); and TRAC Laboratories, 113 South Cedar, Denton, Texas 76201 (B.V.)

***N*-Acylethanolamines (NAEs) were quantified in seeds of several plant species and several cultivated varieties of a single species (cotton [*Gossypium hirstutum*]) by gas chromatography-mass spectroscopy. The total NAE content of dry seeds ranged from 490 ± 89 ng g^{-1} fresh weight in pea (*Pisum sativum* cv early Alaska) to 1,608 ± 309 ng g^{-1} fresh weight in cotton (cv Stoneville 7A glandless). Molecular species of NAEs in all seeds contained predominantly 16C and 18C fatty acids, with *N*-linoleoylethanolamine (NAE18:2) being the most abundant (approaching 1,000 ng g^{-1} fresh weight in cottonseeds). Total NAE levels dropped drastically following 4 h of imbibition in seeds of pea, cotton, and peanut (*Arachis hypogea* cv Virginia), and this decline was most pronounced for NAE18:2. A novel enzyme activity was identified in cytosolic fractions of imbibed cottonseeds that hydrolyzed NAE18:2 in vitro. NAE degradation was optimal at 35°C in 50 mM MES buffer, pH 6.5, and was inhibited by phenylmethylsulfonyl fluoride and 5,5′-dithio-bis(2-nitrobenzoic acid), which is typical of other amide hydrolases. Amidohydrolase activity in cytosolic fractions exhibited saturation kinetics toward the NAE18:2 substrate, with an apparent K_m of 65 μM and a V_{max} of 83 nmol min^{-1} mg^{-1} protein. Total NAE amidohydrolase activity increased during seed imbibition, with the highest levels (about four times that in dry seeds) measured 2 h after commencing hydration. NAEs belong to the family of "endocannabinoids," which have been identified as potent lipid mediators in other types of eukaryotic cells. This raises the possibility that their imbibition-induced metabolism in plants is involved in the regulation of seed germination.**

NAPE is a membrane phospholipid of plant and animal cells with at least two proposed functional roles (Schmid et al., 1996): (a) to support the structural integrity of biomembranes, and (b) to act as a precursor for the production of lipid mediators. The existence of NAPE in higher plants was the subject of much controversy until a combination of biochemical and biophysical experimental evidence established unequivocally its natural occurrence in a wide range of plant tissues (Chapman and Moore, 1993). NAPE is not abundant under normal physiological conditions. For example, in cotton (*Gossypium hirsutum*) plants, NAPE content varies between 1.9 and 3.2 mol % of the total phospholipid, depending upon the tissue source and developmental stage (Sandoval et al., 1995; Chapman and Sprinkle, 1996). In mammals, NAPE accumulates only in membranes of damaged cells during tissue injury to about 10 mol % of the total phospholipid (Schmid et al., 1990). Biophysical studies indicate that NAPE is a bilayer-stabilizing lipid (LaFrance et al., 1997), and this has prompted some to speculate that it may be synthesized under stress conditions to help maintain membrane integrity and minimize cellular injury. In plants, NAPE is synthesized from two potential bilayer-destabilizing lipids—free fatty acids and PE (McAndrew and Chapman, 1998)—and so under certain conditions, NAPE biosynthesis may have a protective role in plant membranes.

NAPE is an *N*-acylated derivative of the common membrane phospholipid PE and is metabolized by a phosphodiesterase (of the PLD type) to yield phosphatidic acid and NAE (Chapman et al., 1995; Schmid et al., 1996; Chapman, 1998). NAPE is believed to be the precursor in vivo for the entire family of bioactive NAEs. In mammalian neurons, anandamide (*N*-arachidonylethanolamine) is an endogenous ligand for the cannabinoid receptor (for review, see DiMarzo, 1998) and is produced from *N*-arachidonyl PE by a Ca^{2+}-stimulated, PLD-type activity (Cadas et al., 1997). We recently identified a signal-mediated release of NAE from NAPE in elicitor-treated tobacco cell suspensions (Chapman et al., 1998) and leaves (S. Tripathy, B. Venables, and K.D. Chapman, unpublished results). These NAEs were identified by GC-MS as *N*-lauroyl- and *N*-myristoylethanolamine, and an enzyme activity was identified in tobacco microsomes that catalyzed the formation of NAE from NAPE in vitro. Also, homogenates of tobacco cell suspensions hydrolyzed NAE to form free fatty acids and ethanolamine, providing evidence for an intracellular amidohydrolase activity capable of metabolizing NAEs.

In recent years we have accumulated considerable information on the biosynthesis of NAPE during cottonseed development, germination, and seedling growth (Sandoval et al., 1995; Chapman and Sprinkle, 1996). NAPE biosynthesis is increased upon imbibition in cotyledons of cotton,

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* Corresponding author; e-mail chapman@unt.edu; fax 940–565–4136.

Abbreviations: NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PE, phosphatidylethanolamine; PLD, phospholipase D; TMS, trimethylsilyl.

and we were interested in the occurrence and fate of NAE (a presumed NAPE metabolite) in seeds as well. We report the levels of individual NAE molecular species (quantified by GC-MS) among seeds of several plant species and of several cultivars of cotton. NAE levels diminished rapidly in seeds during imbibition, and we identified and partially characterized a novel enzyme in imbibed cottonseeds that hydrolyzed NAE18:2 in vitro. Our results indicate a rapid metabolism of these potentially bioactive lipids during seed imbibition, and may suggest a role for these compounds in germination.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv Long Keeper), pea (*Pisum sativum* cv Early Alaska), castor (*Ricinus communis* cv Zanzabarensis), and peanut (*Arachis hypogea* cv Virginia) seeds were from Gurney's Seed and Nursery (Yankton, SD). Okra (*Abelmoschus esculentus* Moench cv Mammoth Pod) seeds were from plants propagated locally in our greenhouse (in the summer of 1996). Soybean (*Glycine max* cv Dare) seeds were a gift from Dr. Richard Wilson (North Carolina State University, Raleigh). Corn (*Zea mays*) seeds were purchased from Modern Biology (West Lafayette, IN). Cotton (*Gossypium hirsutum*) seeds were from Dr. Rick Turley (U.S. Department of Agriculture-Agricultural Research Service, Stoneville, MS), Dr. John Gannaway (Texas A&M University, Agricultural Experiment Station, Lubbock, TX), or Dr. John Burke (U.S. Department of Agriculture-Agricultural Research Service, Lubbock, TX), and the varieties are listed in Figure 4. All seeds were greater than 90% viable. For imbibition experiments, seeds were surface-sterilized in 10% commercial bleach and soaked in distilled water (in the dark) for 4 h at 30°C with aeration.

NAE Quantification

NAEs were isolated from crude lipid extracts by HPLC and these NAE-enriched fractions were identified and quantified as TMS-ether derivatives by GC-MS (Tripathy et al., 1999). The method is similar to that used by Piomelli and co-workers (Stella et al., 1997) for the analysis of anandamide in mammalian brain extracts, but with some modifications for quantification of unknown plant NAEs in lipid-rich seed extracts. One-gram portions of seeds were powdered in liquid N_2 in a mortar and added to hot 2-propanol (to inactivate any endogenous phospholipases) (Chapman et al., 1998). Lipids were extracted into chloroform, filtered, and subjected to normal-phase HPLC (4.6- × 250-mm Partisil 5 column, Whatman; model 712 HPLC system, Gilson, Middleton, WI). The lipids were suspended in chloroform (200 μL total volume) and separated with a linear gradient of 2-propanol in hexane (up to 40% 2-propanol over 20 min), followed by 5 min at 50% 2-propanol, and then 5 min at 100% hexane. Under these conditions NAEs eluted between 11 and 15 min (at about 30% 2-propanol in hexane), depending on the species, well away from most other lipids (see Fig. 1 for representative traces). A synthetic standard NAE20:4 with substantial UV absorbance at 214 nm was used to monitor NAE retention times and column performance on a daily basis.

The NAE-enriched HPLC fractions were collected, evaporated to dryness under N_2 gas, and derivatized in bis(trimethylsilyl)trifluoroacetamide at 50°C for 30 min. TMS-ether derivatives were suspended in hexane and analyzed by GC-MS. See Figure 2 for representative electron impact mass spectra of NAE18:2 identified in pea extracts and in the NAE18:2 synthetic standard. The GC (model 5890 series II, Hewlett-Packard) was equipped with a capillary column (30-m × 0.25-mm i.d. with a 0.25-μm film thickness; model DB-5.625, J&W Scientific, Folsom, CA). The injector temperature was 260°C and the oven temperature was programmed from 40°C to 280°C at 10°C/min.

The GC was coupled to a mass spectrometer (model HP5970, Hewlett-Packard) equipped with an electron impact source (70 eV) and operated for ultimate sensitivity in the selective ion monitoring mode. The $[M]^+$or $[M-15]^+$ ions, as well as two additional confirming masses, were monitored for each NAE species. Standard curves and mass spectra were prepared using injected masses of 0.1 to 30 ng of synthetic NAE from each species (NAE12:0, NAE14:0, NAE16:0, NAE18:0, NAE18:$1^{cis\Delta9}$, NAE18:$2^{cis\Delta9,12}$, NAE18:$3^{cis\Delta9,12,15}$, NAE18:$3^{cis\Delta6,9,12}$, and

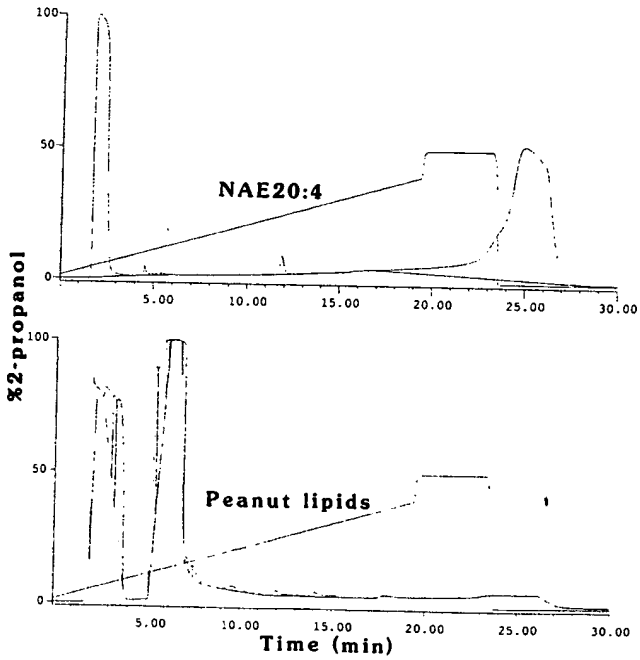

Figure 1. Representative A_{214} profiles (585 mV full scale) of synthetic NAE20:4 (upper trace) and crude peanut lipids (lower trace) fractionated in a 2-propanol gradient (0%–50% in hexane) by HPLC. The peak at approximately 12 min in the upper trace is NAE20:4 (confirmed by GC-MS), the standard that marks the relative retention time of NAEs in these separations. A fraction from 11 to 15 min was collected from HPLC separations of crude seed lipids (see lower trace for example); NAEs are enriched in the 11- to 15-min fraction and the majority of contaminating lipids (mostly triacylglycerols) were removed by 8 min. This represents a major "clean-up" step since, in comparison, peanut seeds contain about 45% oil by weight.

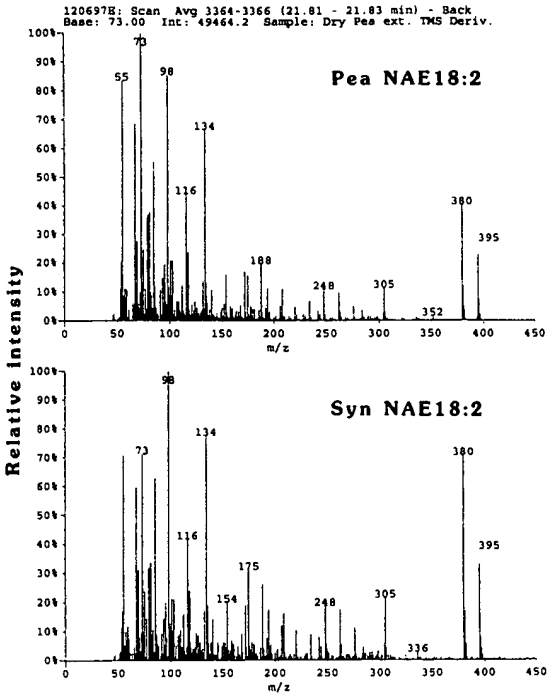

Figure 2. Representative electron impact mass spectra for TMS-ether derivatives of NAE18:2 isolated from pea seeds (upper) and, for comparison, our synthetic NAE18:2 quantitative standard (lower). These compounds have identical retention times on GC (21.82 min; not shown), and their electron impact mass spectra are virtually indistinguishable. Identifiable ions used for quantification purposes include the molecular ion M^+ at *m/z* 395, fragmentation ions $[M-15]^+$ at *m/z* 380, and $[M-90]^+$ at *m/z* 305. For all NAEs in seed extracts that were identified and quantified by GC-MS, identical GC retention times and electron impact mass spectra were obtained authentic synthetic standards (not shown), similar to the above example.

NAE20:4$^{cis\Delta5,8,11,14}$) in the presence of 10 ng of internal standard (decachlorobiphenyl). Final quantification of NAE species was calculated from the ratio of analyte (NAE) response to that of the internal standard. Method efficiency was evaluated by the recovery of NAE17:0 "surrogate" added to the preparation at the time of lipid extraction, and replicate values were adjusted for NAE17:0 recovery.

Synthetic NAE species were prepared from acyl chlorides in ethanolamine essentially as described previously (Devane et al., 1992). Purity and yield were confirmed by GC-MS.

Preparation of Cell Fractions

Imbibed cotton (cv Stoneville 7A glandless) seeds were homogenized in 1:1 (w/v) ratio of fresh weight to medium and fractionated by differential centrifugation as previously described (Chapman and Sriparameswaran, 1997). Fractions were characterized by distribution of marker enzyme activities (Chapman and Sriparameswaran, 1997). The supernatant from the 60-min spin at 150,000*g* was nearly devoid of any membrane markers and was designated the cytosolic fraction.

NAE Degradation in Vitro

An endpoint assay that follows the consumption of exogenously supplied NAE was developed to detect and measure NAE amidohydrolase activity in cottonseed extracts. It is based on the analysis of TMS-ether derivatives of NAE quantified by GC-flame ionization detection following incubation of synthetic NAE with cell fractions. Derivitization and GC conditions were exactly as described above, except the column had a 30-m × 0.32-mm i.d. External standard NAE 17:0 (200 ng) was added after the enzyme reaction was stopped, and the ratio of NAE17:0 standard to substrate was used to calculate the precise amount of NAE substrate remaining. Results from replicate samples were reproducible with this approach, and the accuracy of GC-flame ionization detection was verified with GC-MS. Assays were initiated by the addition of enzyme to NAE substrate, and were suspended by sonica-

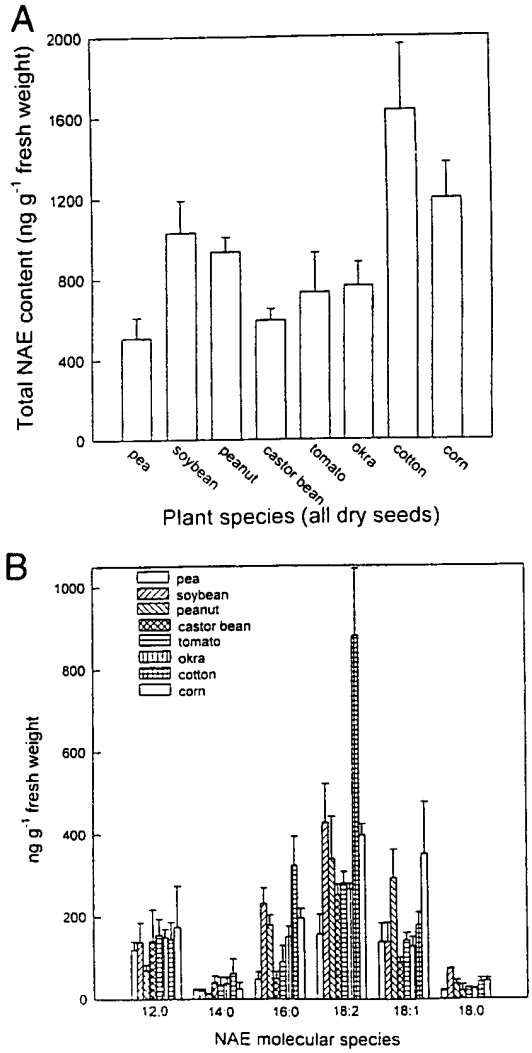

Figure 3. Quantification of NAE in dry seeds of pea, soybean, peanut, castor bean, tomato, okra, cotton, and corn. A, Total NAE content summed from individual molecular species profiles (B). Bars represent the means ± SD of three to six independent extractions and fractionations.

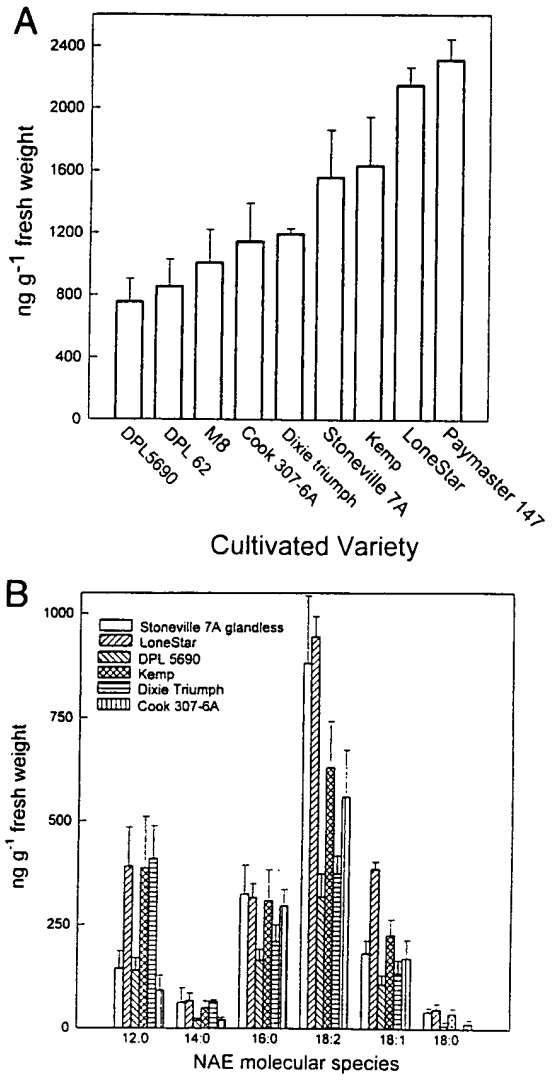

Figure 4. Quantification of NAE in dry seeds of several diverse, cultivated varieties of upland cotton (cvs DPL5690, DPL62, M8, Cook 307–6A, Dixie Triumph, Stoneville 7A glandless, Kemp, LoneStar, and Paymaster 147). A, Total NAE content summed from individual molecular species profiles (B). Bars represent the means ± SD of three to six independent extractions and fractionations.

tion in buffer in a final volume of 0.75 mL. Experiments were conducted in a shaking (60 rpm) bath to determine the optimal time, temperature, protein content, pH, and substrate concentration for amidohydrolase rate measurements. Activity was tested with radiolabeled NAE to confirm conversion of NAE to ethanolamine and free fatty acids (Chapman et al., 1998), and no NAE degradation was detected in the absence of enzyme or with enzyme that had been preincubated for 15 min at 100°C.

RESULTS

NAEs were quantified in lipid extracts from dry seeds of several plant species (Fig. 3). All seeds contained NAEs, but total NAE content varied about 3-fold among the species examined (Fig. 3A). Total NAE content was greatest in cotton (1,608 ± 309 ng g^{-1} fresh weight) and lowest in pea (490 ± 89 ng g^{-1} fresh weight). The individual molecular species of NAE were also analyzed (Fig. 3B). While NAE profiles in all seeds were similar qualitatively, there was considerable quantitative variability (particularly for NAE16:0, NAE18:2, and NAE18:1) among the species, which was not entirely accounted for by differences in total NAE content. NAE18:2 was the most abundant species in seeds, and NAE14:0 and NAE18:0 were the least abundant. This represents a marked difference from profiles of NAEs in leaves, where medium-chain, saturated NAE species were predominant (Chapman et al., 1998; Tripathy et al., 1999; K.D. Chapman and B. Venables, unpublished results). This may suggest distinctly different roles for NAE species in different plant tissues.

Similar to results among seeds of different plant species, we found about a 3-fold difference in total NAE content among several cultivars of cotton (Fig. 4). Total NAE levels ranged from just under 800 ng g^{-1} fresh weight for cv DPL5690 to nearly 2,400 ng g^{-1} fresh weight for cv Paymaster 147. As with seeds of different plant species, NAE18:2 was the most abundant species in the different cotton varieties. Variability in NAE content and acyl composition among cultivars of a single species was nearly as great as the variability among seeds of different species. These data would suggest that the variability is not necessarily physiologically significant and that there are likely cultivars of pea, for example, that have higher NAE levels than some cotton varieties. Perhaps the absolute amount of NAE (above a certain minimum level) in quiescent seeds is less important than its imbibition-induced metabolism (see below).

Because NAPE is the presumed precursor for NAEs, the *N*-acyl compositions of NAPE in dry seeds of peanut, pea,

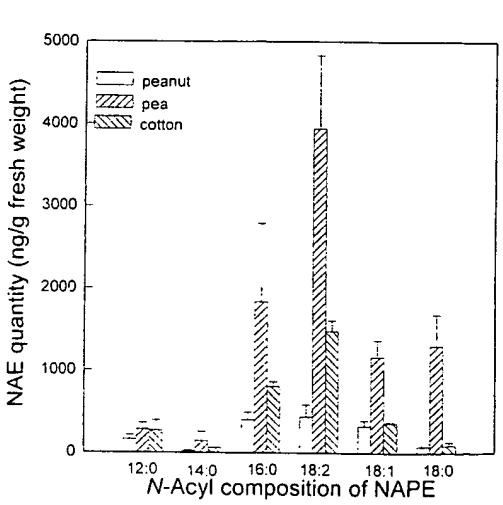

Figure 5. Comparison of the relative abundance of NAE moieties of dry seed NAPE generated enzymatically (see "Materials and Methods"). NAPE was purified by TLC from dry seeds of peanut, pea, and the cotton cv Stoneville 7A glandless, as described previously (Chapman and Moore, 1993). Bars represent the means ± SD of three independent extractions and fractionations.

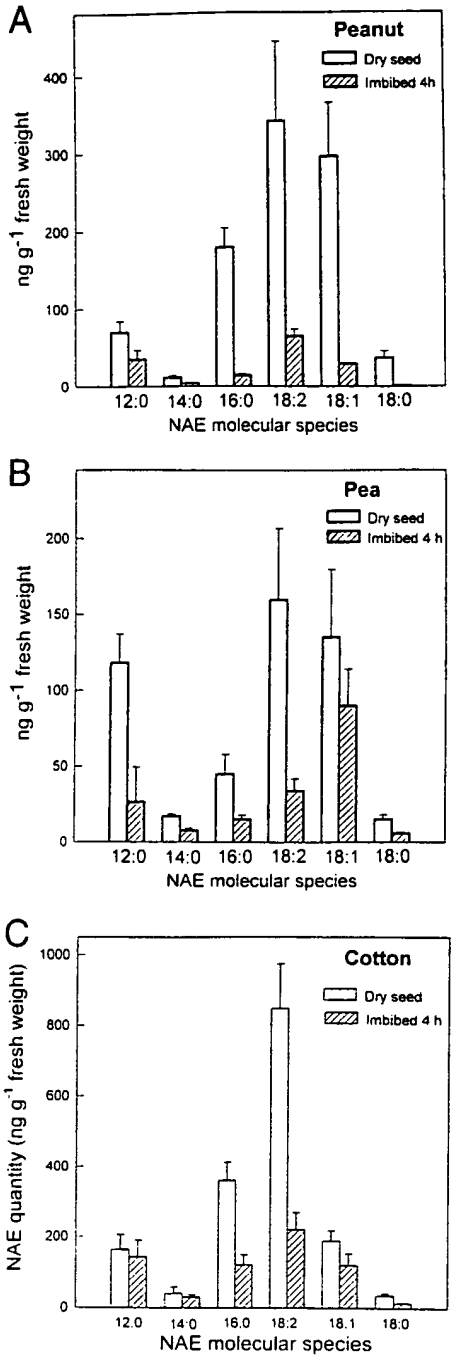

Figure 6. Quantification of NAE molecular species in dry (white bars) and 4-h-imbibed (hatched bars) seeds of peanut (A), pea (B), and the cotton cv Stoneville 7A glandless (C). Bars represent the means ± SD of three to six independent extractions and fractionations.

and cotton were compared (Fig. 5). The *N*-acyl portion of purified NAPE was generated by enzymatic digestion with a *Streptomyces chromofuscus* phosphodiesterase (Chapman and Moore, 1993), and the resulting NAE species were quantified by GC-MS. The same fatty acids that were constituents of seed NAEs (Fig. 3) were present in NAPE (Fig. 5), although their relative abundance was not identical. As expected, NAE18:2 and NAE16:0 were the most abundant

Table I. *Distribution of imbibed cottonseed NAE18:2 amidohydrolase activity in crude cell fractions*

Fractions were prepared in 100 mM potassium-phosphate (pH 7.2), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 400 mM Suc, and assayed for amidohydrolase activity in the same buffer for 10 min at 35°C at a substrate concentration of 60 μM. Clarified homogenate (640*g*, 10 min supernatant) represents total activity. The 10,000*g* pellet is enriched in plastids, mitochondria, and glyoxysomes, while the 150,000*g* pellet is enriched in microsomes membranes derived from ER, Golgi, and plasma membranes (Chapman and Sriparameswaran, 1997). The 150,000*g* supernatant is enriched in cytosolic proteins. Values represent averages of duplicate assays from a single experiment. Similar results were obtained in replicate experiments.

Sample	Total Activity	Specific Activity
	nmol min^{-1}	*nmol min*$^{-1}$ *mg*$^{-1}$ *protein*
Clarified homogenate	307.8	5.1
10,000*g* (30 min) Pellet	17.3	1.2
150,000*g* (60 min) Pellet of a 10,000*g* supernatant	28.2	9.4
150,000*g* (60 min) Supernatant of a 10,000*g* supernatant	223.2	18.1

in NAPE isolated from all three species. For the most part, quantities of NAPE were in excess of the levels of NAEs in these seeds, which is consistent with the notion that NAEs are derived from NAPE. Interestingly, pea seeds contained the most NAPE (nearly 10 μg g^{-1} fresh weight), while they contained the least NAE of the seeds examined. While experimental conditions and analytical procedures were different, our results are consistent with previous findings that NAPE levels are substantial in dry pea seeds (Dawson et al., 1969).

NAE molecular species were quantified in imbibed (4 h) seeds of pea, cotton, and peanut (Fig. 6). Compared with dry seed levels, there was a dramatic reduction in most NAE species in all of these seeds, even considering the

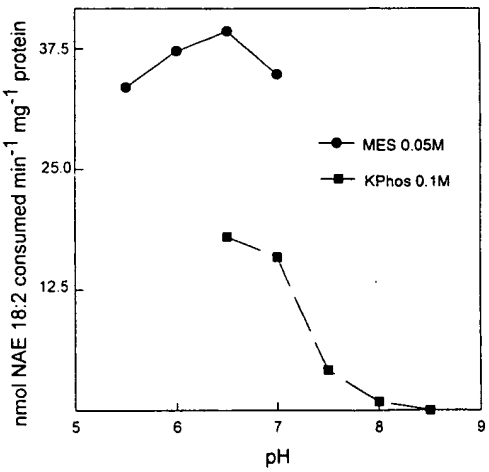

Figure 7. Degradation of NAE18:2 in cytosolic fractions of imbibed cottonseeds at varying pH. Assays were conducted for 10 min at 35°C, and were initiated by the addition of enzyme. Substrate (60 μM NAE18:2) was solubilized in buffer with sonication, and the final assay volume was 0.75 mL. Data points are averages of duplicate assays and are representative of replicate experiments.

Table II. *Influence of divalent cations and Ser and Cys group modifiers on NAE 18:2 degradation in vitro*

Assays were initiated by the addition of enzyme in 50 mM MES buffer, pH 6.5, at 100 μM NAE 18:2. Values are averages of duplicate assays (less than 10% variability) of a single cytosolic preparation. Similar results were obtained in replicate experiments with independently prepared cytosolic fractions.

Sample	NAE 18:2
	nmol consumed min^{-1} mg^{-1} protein
Cytosol[a] only	47.4
Cytosol +1 mM $MgCl_2$	47.7
Cytosol + 1 mM $MgCl_2$ +1 mM EDTA	49.7
Cytosol + 1 mM $MnCl_2$	48.2
Cytosol + 1 mM $MnCl_2$ + 1 mM EDTA	49.3
Cytosol + 1 mM $CaCl_2$	48.1
Cytosol + 1 mM $CaCl_2$ + 1 mM EGTA	47.5
Cytosol + 10 mM PMSF	25.8
Cytosol + 10 mM DTNB	10.8

[a] Cytosol was a 150,000*g*, 60 min supernatant (from a 10,000*g*, 30-min supernatant).

increase in fresh weight contributed by water (e.g. an increase of about 30% fresh weight in 4-h-imbibing cottonseeds). The drop in NAE levels was particularly notable for NAE18:2 and NAE16:0 in pea and cotton, while almost all NAEs were diminished in peanut seeds. Because of the short time period (4 h), and because a decrease in NAEs occurred in both oilseeds and non-oilseeds, it is unlikely that the metabolism of NAEs upon imbibition is associated with general lipid mobilization. Rather, we postulate that the metabolism of NAE is related specifically to the initiation of seed germination.

Exogenously supplied NAE18:2 was hydrolyzed in vitro in cell fractions of imbibed cottonseeds (Table I). NAE hydrolysis was highest in cytosolic fractions (83% of total activity was recovered in the 60-min 150,000*g* supernatant).

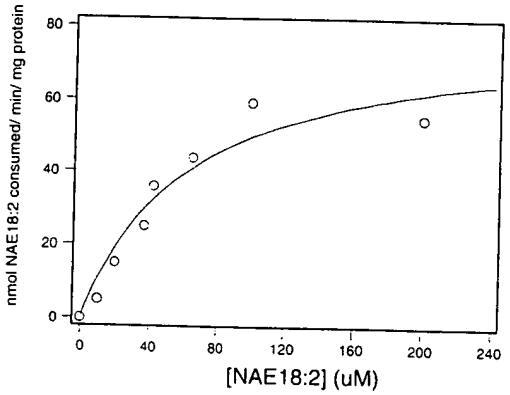

Figure 8. Plot of NAE18:2 degradation versus the concentration of NAE18:2. Assays were for 10 min in 50 mM MES buffer, pH 6.5, and 0.05 mg of cytosolic protein in a final volume of 0.75 mL. Substrate was solubilized in buffer with sonication, and the reaction was initiated by the addition of enzyme. Data points are averages of duplicate assays and are representative of replicate experiments. The solid line represents the data fit to the Michaelis-Menten equation (MacCurveFit software, produced by Kevin Reiner).

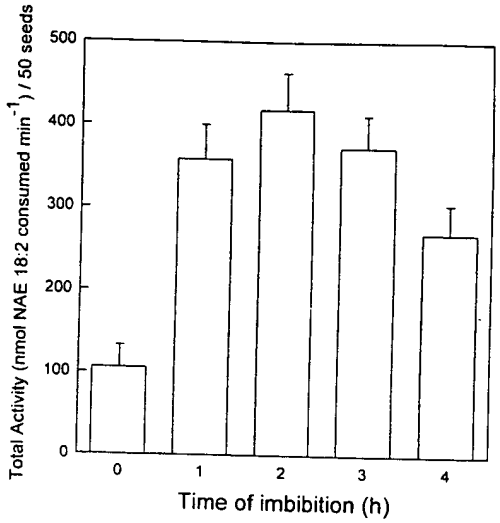

Figure 9. Time course of NAE 18:2 amidohydrolase activity measured in vitro in cell fractions (10,000*g*, 30-min supernatant) from dry or imbibed (for 1–4 h) cottonseeds. Results were calculated as total activity in extracts from 50 seeds at each time point, and values are the average ± SE from three separate experiments. Assay conditions were as described in the legend for Figure 8, except a final substrate concentration of 100 μM was used and the enzyme content was varied between 20 and 100 μg.

The rate of hydrolysis in cytosolic fractions was linear for 30 min with up to 120 μg of protein and optimal at 35°C (not shown). The reaction was pH dependent, with an optimum of 6.5, and the exchange of MES buffer (50 mM) for potassium-phosphate buffer doubled the rate of NAE degradation (Fig. 7). NAE hydrolysis in vitro apparently was not dependent on Mg^{2+}, Mn^{2+}, or Ca^{2+}, but was inhibited by both PMSF and 5,5′-dithio-bis(2-nitrobenzoic acid) (Table II), which is similar to amidohydrolases that degrade NAEs in mammalian systems (Schmid, 1996).

Hydrolysis of NAE18:2 in vitro was dependent upon substrate concentration, exhibiting typical saturation kinetics (Fig. 8). Fitting the data in Figure 8 to the Michaelis-Menten equation (solid line, $r^2 = 0.94$) gave an apparent K_m and V_{max} of 65 μM and 83 nmol min^{-1} mg^{-1} protein, respectively. The cytosolic fraction exhibited little activity toward NAE14:0 (not shown).

NAE amidohydrolase activity was detected in extracts of dry cottonseeds, and the relative activity increased about four times in just 2 h of imbibition (Fig. 9), suggesting a rapid activation of enzyme activity. A more complete characterization of this NAE amidohydrolase activity will not be not possible until it is purified; however, it is clear that imbibed seeds contain an enzyme(s) capable of hydrolyzing NAE18:2 that is likely responsible for imbibition-induced degradation of NAEs in vivo.

DISCUSSION

Until recently, NAPE was believed by many to be a misidentified artifact of lipid extraction, not an endogenous constituent of plant cells (for discussion, see Chapman and Moore, 1993). However, fast-atom bombardment-

MS/MS approaches helped to establish unequivocally the natural occurrence of NAPE in plants and to identify several molecular species of NAPE in cottonseeds (Chapman and Moore, 1993a; Sandoval et al., 1995). Radiolabeling experiments in vivo with [1,2-^{14}C]ethanolamine demonstrated the capacity of several plant species to synthesize NAPE de novo during postgerminative growth (Chapman and Moore, 1993a). The biosynthesis and turnover (Chapman et al., 1995) of NAPE to form NAE was reconstituted in vitro in microsomal membranes of cotton cotyledons, indicating that the cellular machinery for the metabolism of NAPE was present in germinated seeds. A developmental profile of NAPE biosynthesis indicated that NAPE synthesis was increased in cottonseeds during imbibition and germination (Chapman and Sprinkle, 1996). Perhaps NAPE biosynthesis is increased during seed imbibition/germination to replenish the cellular reserve of NAE precursor.

The *N*-acylation-phosphodiesterase pathway (Schmid et al., 1996) is apparently responsible for the generation of bioactive NAEs in animals (for review, see DiMarzo, 1998). This pathway involves the biosynthesis of a NAPE precursor that is cleaved in a signal-mediated fashion by a PLD-type enzyme to yield NAE. The specific NAE produced likely depends on a number of regulatory factors such as PLD specificity and the *N*-acyl composition of the precursor pool. Recent evidence indicates that NAPE/NAE metabolism is activated in elicitor-treated plant cells (Chapman et al., 1998; Tripathy et al., 1999) for the release of medium-chain, saturated NAEs (e.g. NAE14:0 levels increased from about 6–240 ng g^{-1} fresh weight in cryptogein-treated tobacco leaves). By comparison, NAE levels were considerably higher in quiescent seeds (Figs. 3 and 4), and the molecular species in seeds consist mainly of C16 and C18 fatty acids, with the most prevalent NAE species being the di-unsaturated NAE18:2. It is possible that different NAEs are accumulated at different developmental stages or in different tissues of plants for different purposes. Future work to identify the physiological role of NAPE/NAE metabolism in plants will require accurate quantification of these metabolites under differing physiological conditions, and the results presented here provide the basis for such future studies in seeds.

Most of the NAEs diminished rapidly upon seed imbibition (Fig. 6). Moreover, an active amidohydrolase activity was identified and partially characterized in the cytosolic fractions of imbibed cottonseeds (Tables I and II; Figs. 7 and 8) that hydrolyzed NAE18:2. This amidohydrolase activity was increased during seed imbibition (Fig. 9). The degradation of NAE by an amidohydrolase(s) is the mechanism by which the NAE neurotransmitter anandamide is inactivated following its selective uptake in mammalian neuronal cells (Cravatt et al., 1996; Beltramo et al., 1997). NAE formation and inactivation is emerging as a central signaling pathway in a variety of eukaryotic cell types (Schmid et al., 1996; Chapman, 1998). Therefore, the rapid changes in seed NAE levels raise the possiblity that their metabolism is involved in cell signaling during seed germination. While it is speculation, it is possible that NAE acts as an endogenous inhibitor that must be removed before germination can proceed.

Another possibility is that NAE metabolism is initiated as part of a protective mechanism to minimize imbibition-induced cellular damage. We previously noted that NAPE biosynthesis was increased in imbibing seeds, and proposed that the synthesis of this membrane-stabilizing lipid may be part of an effort to maintain cellular compartmentation during seed rehydration (for discussion, see Sandoval et al., 1995). NAPE is synthesized by a membrane-bound enzyme (designated NAPE synthase) from free fatty acids and PE (McAndrew and Chapman, 1998). Perhaps the NAE amidohydrolase activity provides free fatty acids for the NAPE synthase to allow cells of imbibing seeds to rapidly adjust their NAPE content. In any case, direct evidence of whether NAE is a lipid mediator or if its metabolism serves a protective role will await the ability to manipulate NAE levels in vivo.

NAE18:2 exhibits cannabimimetic properties when administered to animals (for review, see DiMarzo, 1998). However, these effects likely are indirect, because NAE18:2 was shown to competitively inhibit anandamide degradation by the amidohydrolase enzyme in mammalian cells (diTomaso et al., 1996; Maccarrone et al., 1998). Thus, the presence of NAE18:2 (also identified as a lipid constituent of brain) can potentiate the endogenous activity of anandamide. In the present study, NAE18:2 was the most abundant species of NAE in all dry seeds examined, with levels approaching 1 $\mu g\ g^{-1}$ fresh weight in cottonseeds (Fig. 4B). The transient metabolic changes in cellular NAE levels has been shown to influence many physiological processes in vertebrates, including sleep, memory, pain, and immunity (Schmid et al., 1996; DiMarzo et al., 1998). Consequently, seeds may represent a natural source of new cannabimimetic compounds. In fact, identification of NAEs in processed cocoa powder prompted Piomelli and coworkers to propose that these compounds formed the molecular basis for chocolate cravings (diTomaso et al., 1996). The results reported in this manuscript accurately identify and quantify various NAE species in seeds of higher plants and, for the first time to our knowledge, place their metabolism in the physiological context of seed imbibition/germination.

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N-Acylethanolamines in Signal Transduction of Elicitor Perception. Attenuation of Alkalinization Response and Activation of Defense Gene Expression[1]

Swati Tripathy, Barney J. Venables, and Kent D. Chapman*

University of North Texas, Department of Biological Sciences, Division of Biochemistry and Molecular Biology, Denton, Texas 76203–5220 (S.T., K.D.C.); and TRAC Laboratories, 113 S. Cedar, Denton, Texas 76201 (B.J.V.)

In a recent study of *N*-acylphosphatidylethanolamine (NAPE) metabolism in elicitor-treated tobacco (*Nicotiana tabacum* L.) cells, we identified a rapid release and accumulation of medium-chain *N*-acylethanolamines (NAEs) (e.g. *N*-myristoylethanolamine or NAE 14:0) and a compensatory decrease in cellular NAPE (K.D. Chapman, S. Tripathy, B. Venables, A.D. Desouza [1998] Plant Physiol 116: 1163–1168). In the present study, we extend this observation and report a 10- to 50-fold increase in NAE 14:0 content in leaves of tobacco (cv Xanthi) plants treated with xylanase or cryptogein elicitors. Exogenously supplied synthetic NAE species affected characteristic elicitor-induced and short- and long-term defense responses in cell suspensions of tobacco and long-term defense responses in leaves of intact tobacco plants. In general, synthetic NAEs inhibited elicitor-induced medium alkalinization by tobacco cells in a time- and concentration-dependent manner. Exogenous NAE 14:0 induced expression of phenylalanine ammonia lyase in a manner similar to fungal elicitors in both cell suspensions and leaves of tobacco. NAE 14:0, but not myristic acid, activated phenylalanine ammonia lyase expression at submicromolar concentrations, well within the range of NAE 14:0 levels measured in elicitor-treated plants. Collectively, these results suggest that NAPE metabolism, specifically, the accumulation of NAE 14:0, are part of a signal transduction pathway that modulates cellular defense responses following the perception of fungal elicitors.

Several physiological studies of plant-pathogen interactions have established elicitor recognition as the initial source of signal(s) leading to incompatibility and activation of defense mechanisms (Lamb et al., 1989; Dixon and Lamb, 1990; Atkinson, 1993; Dixon et al., 1994; Boller, 1995). Elicitors are pathogen-derived compounds such as oligosaccharides, glycopeptides/glycoproteins, peptides/proteins, and/or lipids that trigger multiple defense responses (Ebel and Scheel, 1992). The earliest responses of cells in perception of elicitors include the activation of Ca^{2+} influx and K^+/H^+ exchange at the plasma membrane (Atkinson et al., 1985, 1990; Baker et al., 1991). These rapid changes in ion flux are followed by the oxidative burst (Adam et al., 1989; Keppler et al., 1989; Mehdy, 1994; Levine et al., 1994; Lamb and Dixon, 1997; Alvarez et al., 1998) and alteration of the phosphorylation status of proteins (Grab et al., 1989; Dietrich et al., 1990; Felix et al., 1991, 1993; Suzuki and Shinshi, 1995; Xing et al., 1996; Adam et al., 1997). These early events constitute a signaling pathway that leads to transcriptional activation of defense gene expression (Cramer et al., 1985; Lawton and Lamb, 1987; Suzuki et al., 1995; Jabs et al., 1997; Chamnongpol et al., 1998).

Among the best-characterized of plant defense genes are those encoding phenylalanine ammonia lyase (PAL), a key regulatory enzyme in phenylpropanoid metabolism (Lamb et al., 1989; Bowles, 1990; Dixon and Paiva, 1995). Several endogenous transmittable signals, such as salicylic acid, systemin, jasmonic acid, and ethylene, that are increased in plant cells following elicitor treatment or pathogen attack, can induce PAL expression (for review, see Enyedi et al., 1992) as part of a localized host defense-response. Recently, a pathogen-induced NO-signaling pathway was identified in plants (Delledonne et al., 1998), that involves production of cGMP and cADP-Rib as second messengers (Durner et al., 1998), and this pathway appears to selectively activate PAL expression.

Over the past several years a number of studies (Anderson et al., 1990; Lotan and Fluhr, 1990; Felix et al., 1993; Moreau et al., 1994) have characterized the multiple cellular responses of tobacco (*Nicotiana tabacum* L.) to xylanase (an elicitor protein from *Trichoderma viride*, Dean et al., 1989), including Ca^{2+} influx, K^+/H^+ exchange, induction of ethylene biosynthesis, production of phytoalexins, synthesis of pathogenesis-related proteins, and changes in membrane lipid composition. A plasma membrane receptor for xylanase was recently identified on tobacco cells (Hanania and Avni, 1997), and sensitivity to xylanase was linked to a single gene trait in tobacco (Bailey et al., 1993). In most cases these cellular responses of tobacco to xylanase are characteristic of resistant interactions in host plants.

In the present study we explore at the cellular and intact plant levels the physiological role of medium-chain *N*-acylethanolamine (NAE) accumulation with respect to defense-related signal transduction pathways in tobacco. In two earlier studies, *N*-acylphosphatidylethanolamine (NAPE) metabolism in tobacco cell suspensions was acti-

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* Corresponding author; e-mail chapman@unt.edu; fax 94–565–4136.

vated by the addition of xylanase. NAPE biosynthesis increased 1 to 2 h after xylanase treatment and this was preceded by a rapid hydrolysis of NAPE (Chapman et al., 1995, 1998). A phospholipase D (PLD)-type activity was identified in tobacco membranes that hydrolyzed NAPE to NAE in vitro, and this regulated PLD activity may be attributable to the recently discovered PLD β or γ isoforms (Pappan et al., 1997, 1998). Release and accumulation of NAE (Chapman et al., 1998) has prompted further interest in a possible role for NAE in elicitor-plant interactions.

In animal cells, NAEs and their precursors, NAPEs, have gained renewed interest as bioactive lipid molecules. Long-chain NAEs increased along with the corresponding NAPE under pathophysiological conditions (Epps et al., 1979, 1980; Cadas et al., 1997; Kondo et al., 1998; Sepe et al., 1998) and were involved in changes in membrane function (for review, see Schmid et al., 1990, 1996). The polyunsaturated anandamide (NAE 20:4) is an endogenous ligand for the cannabinoid receptor (CB1) in mammalian brain (Devane et al., 1992; Hanus et al., 1993), and is released from NAPE following signal-mediated activation of PLD (Cadas et al., 1997).

Recently, a number of other biological activities have been attributed to NAEs (mostly to anandamide) in vertebrates, including attenuation of pain (Jagger et al., 1998), embryo implantation (Das et al., 1995), and immunomodulation (for review, see Di Marzo, 1998). In plants, medium-chain, saturated NAEs (e.g. NAE 14:0) accumulated in elicitor-treated tobacco cell suspensions (Chapman et al., 1998). Here we provide evidence that NAE 14:0 at nanomolar concentrations activates PAL expression in cell suspensions and leaves of tobacco, suggesting that NAE release is part of a signal transduction pathway(s) from elicitor perception to PAL expression. Our results also suggest that NAEs may modulate ion flux at the plasma membrane, as indicated by attenuation of elicitor-induced alkalinization of the culture medium. To our knowledge, this represents the first characterization of the biological activity of NAE in plant cells, and extends to plants the role of NAPE/NAE metabolism as a general mechanism for the production of lipid mediators in multicellular eukaryotes.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv KY 14) cell suspensions were grown and maintained as previously described (Chapman et al., 1995) and cell suspensions in log phase (72 h after subculture) were used for elicitor treatments. Cell suspensions were reinitiated from callus cultures periodically (every 3–4 months) to achieve consistent responses to elicitors and NAEs.

Tobacco (cv Xanthi) plants were grown in the greenhouse under a 14-h photoperiod (supplemented with high-intensity sodium lamps when necessary to extend day-length). Fully expanded leaves of 8- to 16-week-old plants were used for experiments.

Elicitor Treatment

Several elicitors of diverse origin were examined, including xylanase (*Trichoderma viride*, Sigma, St. Louis), cryptogein (*Phytopthora cryptogea*, kindly provided by Dr. R.A. Dixon, S.R. Noble Foundation, Ardmore, OK), harpin$_{pss}$ (He et al., 1993) (*Pseudomonas syringae* pv. *syringae*, kindly provided by Dr. J.C. Baker, Molecular Plant Pathology Laboratory, U.S. Department of Agriculture, Agricultural Research Services, Beltsville, MD), and ergosterol, a fungal membrane sterol. All of the elicitors were either infiltrated as aqueous solutions into tobacco leaves or added into aliquots of cell suspensions as aqueous solutions. Ergosterol was dissolved in culture medium with sonication and vortexing prior to addition to cultures.

NAE Treatment

Initially the NAEs 12:0, 14:0, 16:0, 18:0, 18:1, and 20:4 were kindly supplied by Dr. D. Piomelli (University of California, Irvine). Subsequently, we synthesized various NAE molecular species from acyl chlorides in ethanolamine (Devane et al., 1992). NAEs were synthesized in a reaction mixture of 25 mg of respective acylchlorides, 2.5 mL of dichloromethane, and 2.5 mL of ethanolamine (Sigma-Aldrich, Milwaukee, WI) at room temperature for 15 min with gentle swirling. The reaction was stopped with 10 mL of ultrapure water and washed twice with an equal volume of ultrapure water (MilliQUF Plus, Millipore, Bedford, MA). The NAEs were then collected in the organic layer, and the dichloromethane was evaporated under N_2 gas. The NAE species were resuspended in anhydrous methanol, and purity was determined by gas chromatography-mass spectroscopy (GC-MS) (see below). For tobacco leaf infiltration, NAE species were dissolved in water (after removal of organic solvent under N_2) with sonication and vortexing, whereas for treatment of cell suspensions, they were dissolved in culture supernatant prior to treatment.

Measurement of Medium Alkalinization

Aliquots of tobacco cell suspensions (20 mL/3–5 g wet weight) were equilibrated for 20 to 30 min with continuous stirring until a steady pH value was reached. The change in medium pH was monitored with a glass combination electrode ($Ag/AgCl_2$, model 15 pH meter, Fisher Scientific, Houston, TX) for 40 min after the elicitor or NAE treatment.

RNA Isolation and Northern Analysis

Total RNA from tobacco leaves and cell suspensions was isolated according to the single-step guanidinium acid-phenol method of Chomczynski and Sacchi (1987). Prior to RNA isolation, tobacco leaves were treated for 12 h and tobacco cells for 4 h with elicitors and/or NAEs. Tobacco cells were collected by centrifugation at 300*g*. RNA was isolated from cells or leaves that were frozen in liquid N_2 and precipitated by overnight incubation at −20°C with 1

volume of isopropanol. RNA samples (10 μg) were separated in agarose-formaldehyde gels (Sambrook et al., 1989) and incubated for 3 h in hydrolysis buffer (50 mM NaOH and 10 mM NaCl) and for 20 min (2×) in neutralization buffer (0.2 M Tris, pH 7.4, and 18× SSC) prior to blotting (Hybond N+, Amersham-Pharmacia Biotech, Uppsala) through capillary transfer in 20× SSC (3.0 M NaCl and 0.3 M sodium citrate, pH 7.4). Equal loading of samples was confirmed by including ethidium bromide in the gel-loading samples and also by methylene blue staining of blots (Herrin and Schmidt, 1988).

RNA was fixed to membranes by UV cross-linking (5-min exposure, G-30 T 8 UV lamp source, 0.5 m distance). RNA blots were prehybridized at 60°C for 2 h and hybridized (at 60°C for 18–20 h) with a 766-bp PCR fragment of PAL sequence amplified from tobacco (gift of R.A Dixon, S.R. Noble Foundation, Ardmore, OK) by using specific oligonucleotide primers complimentary to a portion of PAL cDNA (GenBank accession no. X78269) (at position +277, the forward primer, 5′-AAAAATGGCTGGTGTTGCACAA-3′ and at +1,052 bp, the reverse primer, 5′-CCATTCACAAGNGCAAGNCCTTCCTTAGG-3′). The PCR fragments were labeled directly (flourescein-11-dUTP) with a random prime labeling module (Gene Images, Amersham, Buckinghamshire, UK) and detected by the detection module (CDP-Star, Gene Images) following the manufacturer's instructions. Relative levels of PAL mRNA were estimated by normalizing to 28S RNA (in the same lanes) using scanning densitometry and the public domain NIH Image program (version 3.1, developed at the United States National Institutes of Health and available at http://rsb.info.nih.gov/nih-image).

NAE Quantification

Previous methods employed for NAE identification and quantification in cell suspensions were inadequate for analysis of more complex tissues of higher plants. Consequently, we adopted a new procedure for the routine identification and quantification of NAE species from tobacco leaves. Analysis of NAE relied on HPLC isolation of NAE-enriched fractions from crude lipid extracts, and the subsequent identification/quantification of trimethyl silyl-derivatized NAEs by GC-MS. This method is similar to that published by Piomelli and co-workers (Stella et al., 1997) for the analysis of anandamide in mammalian brain extracts, but with some modifications for quantification of saturated, medium-chain NAEs in chlorophyll-containing extracts. Following infiltration, tobacco leaves were harvested and immediately frozen (portions of approximately 1.0 g), powdered in liquid N_2 in a mortar, and added to hot 2-propanol (to inactivate any endogenous phospholipases) (Chapman et al., 1998). Lipids were extracted into chloroform, filtered, and subjected to normal phase HPLC (4.6 × 250 mm Partisil 5 column, Whatman, Clifton, NJ). HPLC conditions involved a linear gradient of 2-propanol in hexane (up to 40% 2-propanol over 20 min), followed by 5 min at 50% 2-propanol, and then 5 min at 100% hexane. Under these conditions, NAEs eluted between 11 and 15 min, depending on the species, well away from most other lipids. A synthetic standard NAE 20:4, which has substantial UV absorbance (at 214 nm), was used to check column performance and NAE retention time on a daily basis.

The NAE-enriched HPLC fractions were collected and evaporated to dryness under N_2 gas. NAEs were derivatized in bis(trimethylsilyl)trifluroacetamide at 50°C for 30 m.n. Trimethyl silyl-ether derivatives were suspended in hexane and analyzed by GC-MS. The gas chromatograph was a 5890 series II (Hewlett-Packard, Palo Alto, CA) equipped with a capillary column (30-m × 0.25-mm i.d. with a 0.25-μm film thickness, DB-5.625, J&W Scientific, Folsom, CA). The injector temperature was 260°C and the oven temperature was programmed from 40°C to 280°C at 10°C min^{-1}. The GC was coupled to a mass spectrometer (model HP5970, Hewlett-Packard) equipped with an electron impact source (70 eV) and operated for ultimate sensitivity in the selective ion monitoring mode. The M^+ and $[M-15]^+$ fragmentation ions as well as two confirming masses were monitored for NAE 14:0. Standard curves and mass spectra were prepared using injected masses of 0.1 to 200 ng of synthetic NAE in the presence of 10 ng of internal standard (decachlorobiphenyl). Final quantification of NAE species was calculated from the ratio of analyte (NAE) response to that of the internal standard. Method efficiency was evaluated by recovery of synthetic NAE 17:0 "surrogate" added to the preparation at the time of tissue extraction, and replicate values were adjusted for NAE 17:0 recovery. Statistical comparisons of the data were made using an unpaired Student's *t* test.

RESULTS

Attenuation of Xylanase-Induced Alkalinization by NAEs in Cultured Tobacco Cells

Xylanase induces extracellular medium alkalinization in cell suspension cultures of different plant species within minutes of treatment (Bailey et al., 1992; Felix et al., 1993). The addition of xylanase (1 μg mL^{-1}) to tobacco cells triggered the extracellular pH to change rapidly (0.2–0.5 unit over 40 min, see Fig. 1). Since NAEs are released into tobacco cell culture medium within 10 min of xylanase treatment (Chapman et al., 1998), the effect of NAEs on this xylanase-induced alkalinization was analyzed. Several saturated and unsaturated species of NAE (12:0, 14:0, 16:0, 18:0, 18:1, and 20:4) at 100 μM were added either separately or in conjunction with xylanase to the cell suspensions. All of the NAEs inhibited the xylanase-induced alkalinization of the culture medium (Fig. 1). Of all of the NAEs tested, NAE 12:0 appeared to be the least effective in antagonizing the xylanase-induced alkalinization response. When added alone, the NAEs generally did not affect the medium pH, and results were comparable to control treatments with medium alone. In tobacco, NAE 14:0 was identified as a predominant endogenous NAE (Chapman et al., 1998), so the effect of this species was characterized in more detail in subsequent experiments.

To analyze whether the inhibitory action of NAE 14:0 was elicitor specific or a more general phenomenon, other elicitors were tested (Fig. 2). The bacterial protein harpin

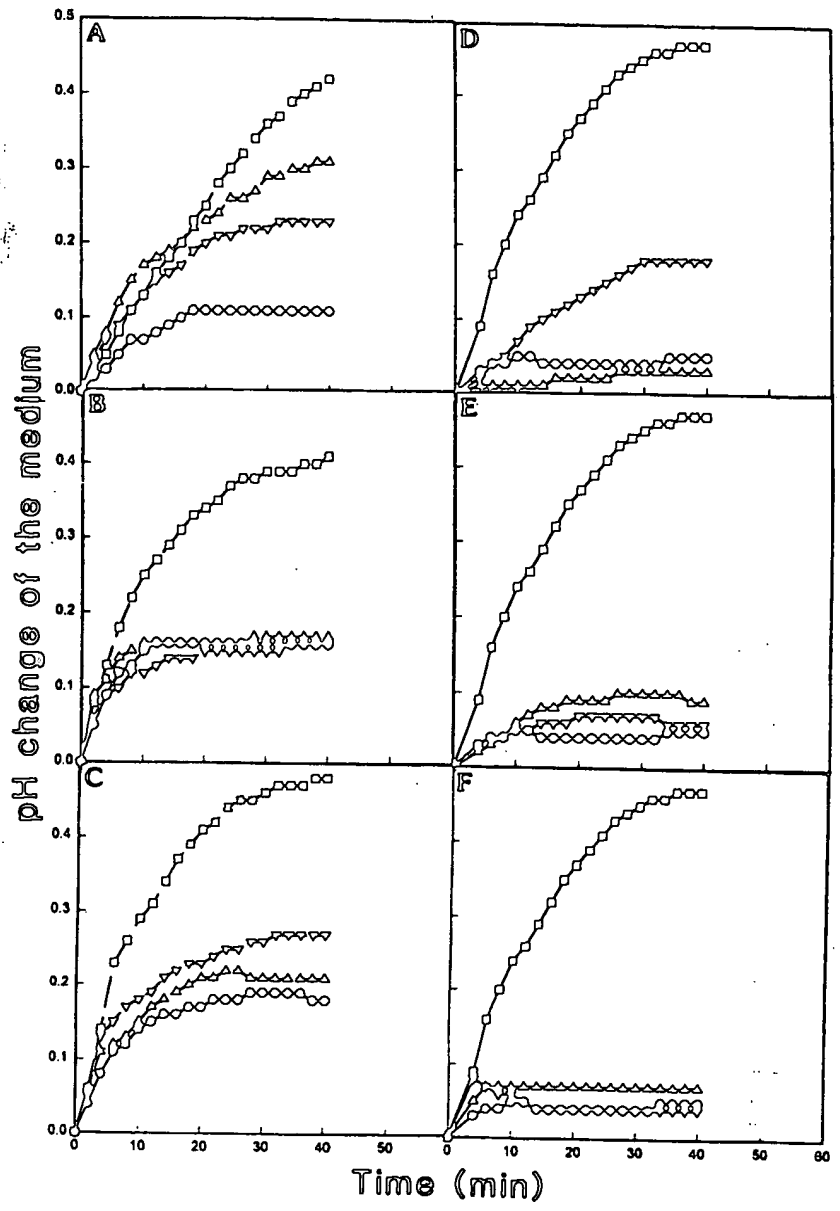

Figure 1. Effects of exogenously supplied synthetic NAEs (0.1 mM) on alkalinization of the tobacco cell culture medium induced by xylanase (1 μg mL^{-1}). The specific NAE molecular species tested were: NAE 12:0 (A); NAE 14:0 (B); NAE 16:0 (C); NAE 18:0 (D); NAE 18:1 (E); and NAE 20:4 (F). NAEs were added to cultures as described in "Materials and Methods." Cells in log phase (3–4 d after subculture) were treated with or without xylanase and incubated with or without NAE with continuous gentle stirring. The change in pH of the culture medium was recorded every 2 min for 40 min. Controls and experimental treatments were carried out on the same population of cells. Results presented are representative; similar trends were observed in experiments repeated three to six times. A, □, xylanase; △, xylanase plus NAE12:0; ▽, NAE12:0; ○, control. B, □, xylanase; △, xylanase plus NAE14:0; ▽, NAE14:0; ○, control. C, □, xylanase; ▽, xylanase plus NAE16:0; △, NAE16:0; ○, control. D, □, xylanase; ▽, xylanase plus NAE18:0; ○, control; △, NAE18:0. E, □, xylanase; △, xylanase plus NAE18:1; ▽, NAE18:1; ○, control. F, □, xylanase; △, xylanase plus NAE20:4; ○, control; ▽, NAE20:4.

(*P. syringae*) and the fungal protein cryptogein (*Phytophthora cryptogea*), both known to activate the alkalinization response in tobacco cell suspensions (Wei et al., 1992; Blein et al., 1991, respectively), and the fungal sterol, ergosterol, which elicits medium alkalinization in tomato cell suspensions (Granado et al., 1995), were tested with NAE 14:0. All of the elicitors induced medium alkalinization (between 0.15–0.4 pH unit), with harpin being the most pronounced, followed by cryptogein and ergosterol (Fig. 2). When NAE 14:0 at 100 μM was included in the treatment along with the elicitor, there was complete inhibition of medium alkalinization in harpin- and cryptogein-treated cell suspensions (Fig. 2, A and B), while inhibition was less obvious in ergosterol-treated cells (Fig. 2C). These results were consistent with the xylanase data (Fig. 1) and suggest that the inhibitory action of NAE can be extended to other elicitors of fungal and bacterial origin.

Time and NAE 14:0 Concentration-Dependent Inhibition of Elicitor-Induced Medium Alkalinization

There was a defined time period in which NAE addition was effective in inhibiting elicitor-induced medium alkalinization (Fig. 3). NAE 14:0 inhibited xylanase-induced alkalinization when added 10 min prior to or at the same time as xylanase. Adding NAE 14:0 10 min after elicitor treatment was marginally effective, if at all, and did not reverse the alkalinization response. Inhibition by NAE 14:0 was concentration dependent, with some inhibition still at 10^{-7} M (Fig. 4A). At lower NAE 14:0 concentrations, a longer time was required to reach 50% inhibition (e.g. at 10^{-7} M, 22 min), emphasizing that the inhibitory effect of NAE was time and concentration dependent. Complete inhibition of the elicitor-induced alkalinization response by NAE is likely a manifestation of higher levels of exogenous

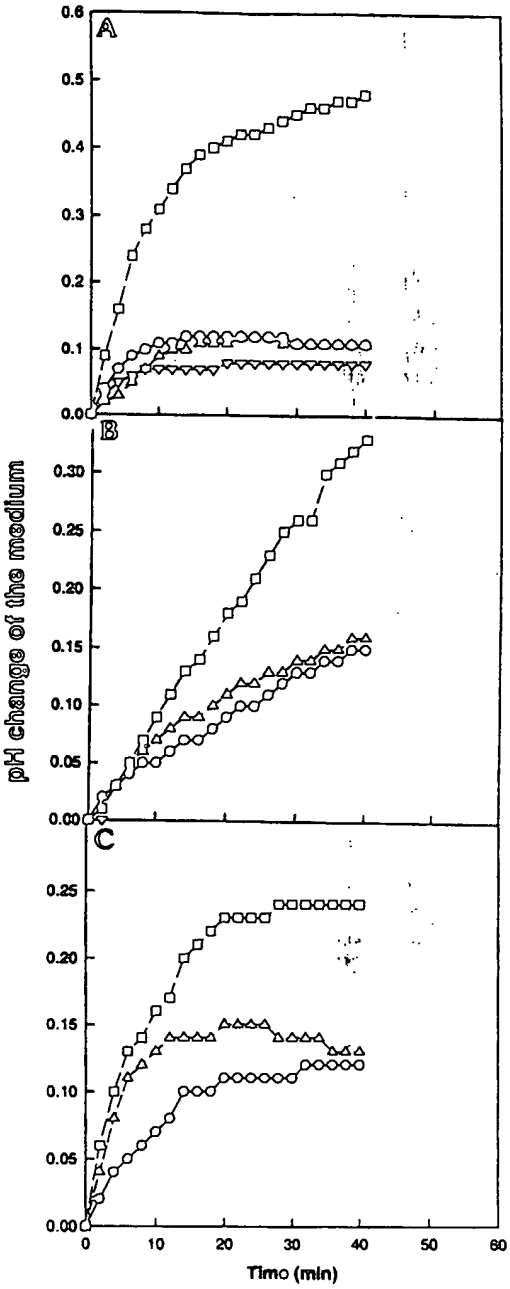

Figure 2. Effect of exogenously supplied NAE 14:0 (0.1 mM) on the alkalinization of tobacco cell culture medium induced by different elicitors. A, Harpin 420 ng mL^{-1} (□), control (○), hairpin plus NAE14:0 (△), and NAE14:0 (▽). B, Cryptogein 150 nM (□), cryptogein plus NAE14:0 (△), and control (○). C, Ergosterol 10 nM (□), ergosterol plus NAE14:0 (△), and control (○). The elicitors and NAEs were added to the culture medium as described in "Materials and Methods" and the pH was recorded every 2 min for 40 min. Controls and experimental treatments were carried out on the same population of cells. Results presented are representative; similar trends were observed in experiments repeated three times.

NAE added at time of elicitor treatment. Endogenous levels of NAE are in the low- to mid-nanomolar range following elicitor treatment, and this may be responsible for the observed attenuation of elicitor-induced medium alkalinization that occurs between 20 and 40 min after elicitor treatment (elicitor only, Figs. 1 and 2). A clear answer will await the ability to block NAE release in vivo. Nonetheless, these results raise the possibility that endogenous release of NAE 14:0 may modulate the well-characterized elicitor-induced exchange response in vivo.

Induction of Defense Gene Expression by Elicitors and NAE 14:0

The effect of NAE 14:0 on PAL gene expression in these tobacco cell suspensions was striking. As expected, xylanase was found to induce PAL gene expression (Fig. 5). Relative PAL expression appeared to be greater when tobacco cells were treated with both xylanase and NAE 14:0 (100 μM). More importantly, PAL expression was induced by NAE 14:0 alone, and this induction was comparable to the relative expression levels induced by xylanase alone. Control cells treated with medium alone (lane 1) did not show any detectable PAL expression.

Since results in cell culture systems are sometimes inconsistent with responses in planta, the same experiments were performed with leaves of tobacco plants (Fig. 6). Tobacco leaves were infiltrated adaxially with xylanase and/or NAE 14:0, and total RNA was isolated after 12 h to analyze PAL expression. Unlike in cell cultures, there was no recognizable additive effect of xylanase and NAE 14:0. However, both xylanase and NAE 14:0 reproducibly activated PAL expression in leaves compared with controls (water only). NAE 14:0 activated PAL expression at concentrations down to 0.1 μM (Fig. 7), similar to treatments

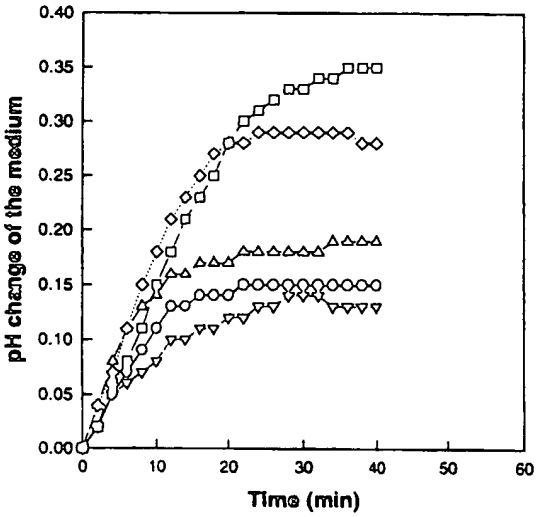

Figure 3. Effect of NAE 14:0 (0.1 mM) on medium alkalinization prior to or during xylanase treatment. NAE 14:0 was added either 10 min before or after xylanase treatment (1 μg mL^{-1}) and the pH of the medium was recorded every 2 min for 40 min. Controls and experimental treatments were carried out on the same population of cells. Results are representative of trends observed in two replicate experiments. □, Xylanase; ◇, NAE14:0 added 10 min after xylanase; △, xylanase plus NAE14:0 added at the same time; ○, control; ▽, NAE14:0 added 10 min before xylanase.

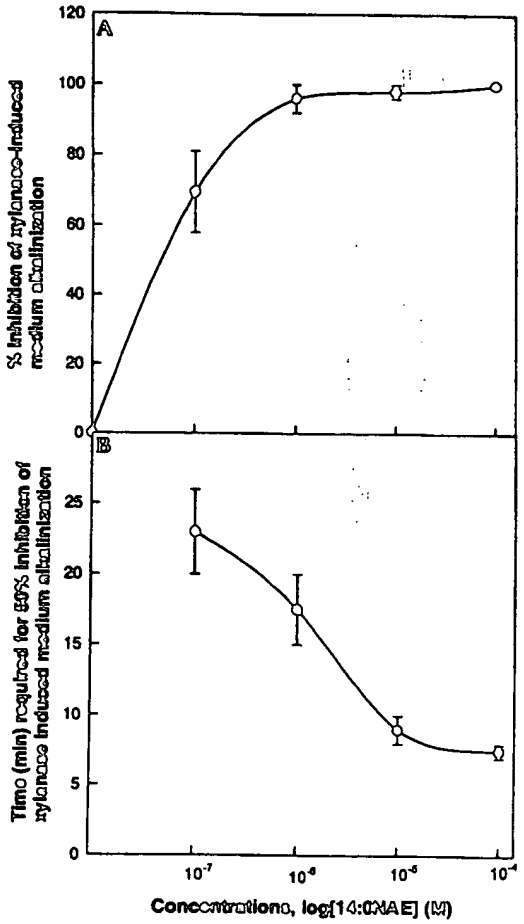

Figure 4. Time- and concentration-dependent inhibition of the xylanase-induced alkalinization of tobacco cell culture medium by NAE 14:0. In A, the percent of overall inhibition (relative to medium controls) versus the log of the concentration of exogenously supplied NAE 14:0 is plotted. In B, the time required to achieve 50% inhibition versus the log of the concentration of exogenously supplied NAE 14:0 is plotted. The data points represent the averages ± SD of three independent experiments at each concentration.

with xylanase or cryptogein. Moreover, the analogous fatty acid myristic acid showed no activation of PAL expression even at 100 μM (Fig. 7), ruling out nonspecific detergent effects of lipid treatments in these experiments.

Accumulation of NAE 14:0 in Leaves

NAE 14:0 levels increased 10- to 50-fold in elicitor-treated tobacco leaves compared with leaves infiltrated with water only (Fig. 8). In leaves infiltrated for 10 min with xylanase, NAE 14:0 content increased from 6 ± 4 to 64 ± 29 ng g^{-1} fresh weight (n = 3; $P < 0.03$). In leaves infiltrated for 10 min with cryptogein, NAE 14:0 content increased from 6 ± 4 to 238 ± 35 ng g^{-1} fresh weight (n = 3; $P < 0.0004$). These results indicate that significant increases in NAE 14:0 content occur in elicitor-treated tobacco leaves, consistent with our prior observations in tobacco cell suspensions (Chapman et al., 1998). Increases in NAE 12:0 (previously seen with cell suspensions) were not particularly evident in leaves of intact plants (not shown). These results indicate that two well-characterized elicitors of tobacco defense responses trigger an accumulation of NAE 14:0 in vivo through a range sufficient to activate PAL expression (Fig. 7).

Cryptogein, as expected, also activated PAL expression in tobacco leaves (Fig. 7). Application of both xylanase (Bailey et al., 1990) and cryptogein (Ricci et al., 1989) to tobacco leaves induces development of lesions characteristic of the hypersensitive response that is commonly observed in resistant host-pathogen interactions. Our results indicate that NAPE metabolism is activated by these pathogen elicitors, and consequently imply that NAE release may be part of the initial signaling cascade that ultimately leads to plant disease resistance.

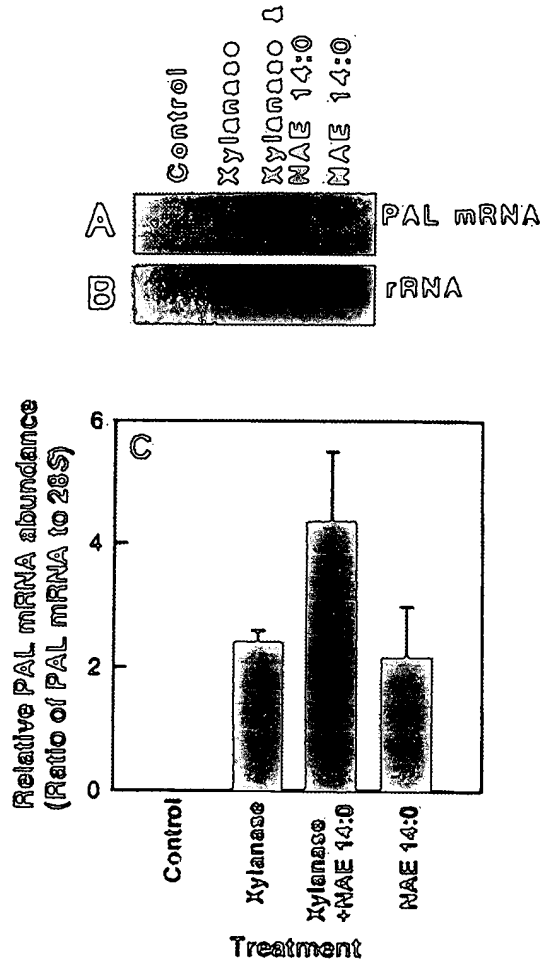

Figure 5. Analysis of PAL mRNA expression in tobacco cell suspensions. A, Northern blot showing PAL expression in cell suspensions treated with medium only (control), xylanase (1 μg mL^{-1}), xylanase in combination with NAE 14:0 (0.1 mM), and NAE 14:0 alone (0.1 mM). B, Methylene blue-stained blot showing relative amounts of RNA in each lane. C, Relative abundance of PAL mRNA (normalized to 28S rRNA by densitometric scanning and imaging analysis with NIH Image 3.1 software). Values represent the means ± SD of three independent experiments/extractions analyzed under identical conditions.

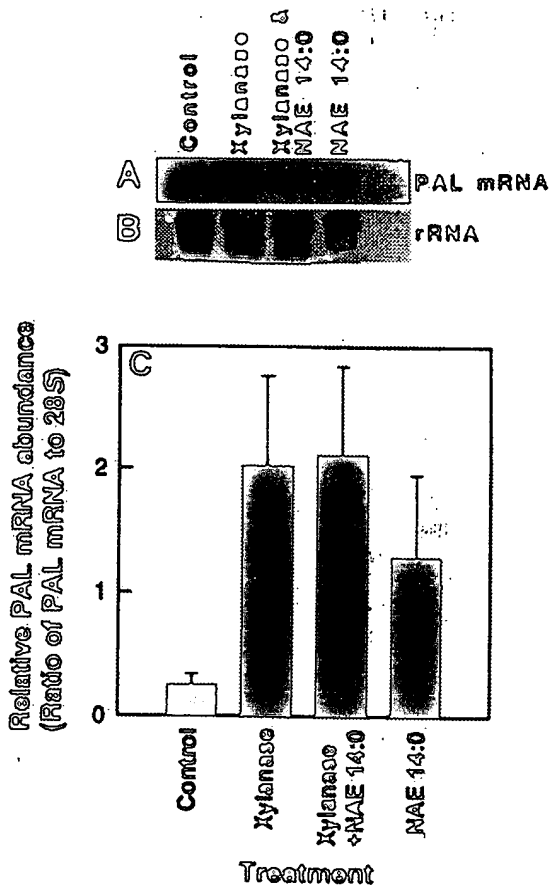

Figure 6. Analysis of PAL mRNA expression in tobacco leaves. A, Northern blot showing PAL expression in leaves treated with water only (control), xylanase (1 μg mL^{-1}), xylanase in combination with NAE 14:0 (0.1 mM), and NAE 14:0 alone (0.1 mM). B, Methylene blue-stained blot showing relative amounts of RNA in each lane. C, Relative abundance of PAL mRNA (normalized to 28S rRNA by densitometric scanning and imaging analysis with NIH Image 3.1 software). Values represent the means ± SD of three independent experiments/extractions analyzed under identical conditions.

DISCUSSION

Membrane phospholipids are the precursors for second messengers produced through transmembrane signaling of activated ion channels, receptor kinases, or through receptor-activated effector enzymes (Munnik et al., 1998). Several phospholipases are known to be activated as a direct consequence of plant-pathogen interactions (for review, see Chapman, 1998). In tobacco cell suspensions, NAE 14:0 (and NAE 12:0) was formed from NAPE (likely by a PLD-type activity) following xylanase treatment and these NAEs accumulated extracellularly (Chapman et al., 1998). Here we demonstrate that NAE 14:0 levels also are increased in tobacco leaves in response to two different pathogen elicitors (Fig. 8). Our results are reminiscent of PLD-mediated NAE release in mammalian systems, and suggest that the *N*-acylation phosphodiesterase pathway (Schmid et al., 1996) operates in plant defense signaling.

Collectively, our results suggest that endogenous NAE (specifically NAE 14:0) acts as a lipid mediator in elicitor-induced cell signaling, and these results can be summarized as follows: (a) a 10- to 50-fold increase in NAE14:0 content was measured (by GC-MS) in planta in response to two different pathogen elicitors (Fig. 8), extending previous observations with cell suspensions (Chapman et al., 1998); (b) six different species of exogenous NAE (including NAE14:0) inhibited elicitor-induced medium alkalinization (Fig. 1); (c) this inhibitory effect was dependent on time and concentration of added NAE (Fig. 3), and was consistently observed for three different elicitors (Fig. 2); (d) NAE 14:0 alone was sufficient to activate PAL expression (in cell suspensions, Fig. 5, and in planta, Fig. 6) in a

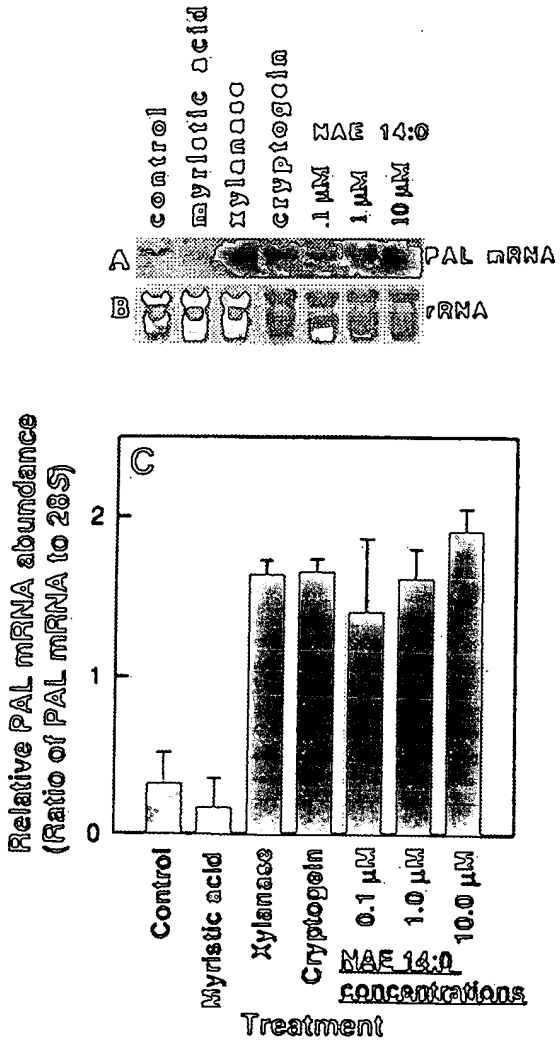

Figure 7. Analysis of PAL mRNA expression in total RNA samples extracted from tobacco leaves treated with various amounts of NAE 14:0. Xylanase and cryptogein were included as positive controls. NAE 14:0 concentrations were varied from 0.1 to 10 μM and myristic acid, a 14:0 fatty acid, was tested at 100 μM. A, Northern blot showing PAL mRNA expression. B, Methylene blue-stained blot showing relative amounts of total RNA in each lane. C, Relative abundance of PAL mRNA (normalized to 28S rRNA by densitometric scanning and imaging analysis with NIH Image 3.1 software). Values represent the means ± SD of three independent experiments/extractions analyzed under identical conditions.

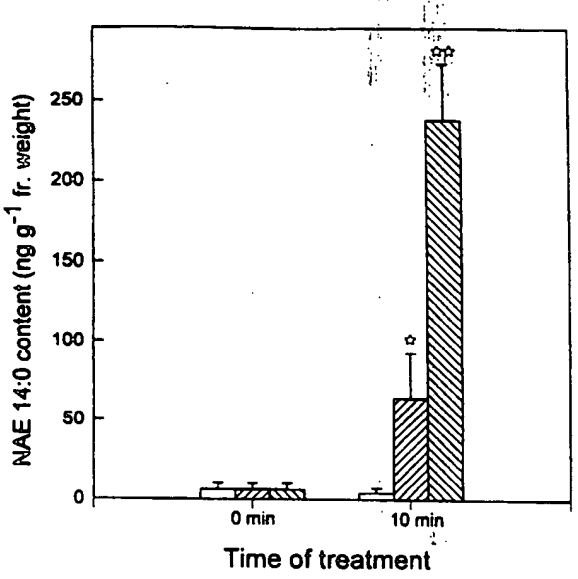

Figure 8. Analysis of elicitor-induced NAE 14:0 content in cv Xanthi tobacco leaves as quantified by GC-MS. Tobacco leaves were infiltrated with xylanase (right-hatched bars; 1 $\mu g\ mL^{-1}$), cryptogein (left-hatched bars; 150 nM), or water (white bars) as described in "Materials and Methods." Each value represents the mean ± SD of three independent experiments and the asterisks (*) indicate statistically significant differences from controls (*, $P < 0.03$; **, $P < 0.0004$).

manner similar to that of the same elicitors that invoked NAE production in vivo; (e) NAE activated PAL expression at submicromolar levels well within the levels we actually quantified in vivo following elicitor treatment, and (f) myristic acid (14:0 fatty acid with no *N*-linked ethanolamine moiety) was inactive in terms of induction of PAL expression. In conclusion, NAEs, an endogenous family of lipid mediators in vertebrates, appear to have a related function in plant cell signal transduction. Future work is aimed at addressing the precise mechanisms of NAE action.

In this manuscript we examined the biological activity of NAE during elicitor perception and plant defense responses. In tobacco cells, attenuation of elicitor-induced medium alkalinization by NAE 14:0 as well as other NAE species suggests an involvement in the modulation of ion flux at the plasma membrane. Interestingly, in animals NAE inhibited the permeability-dependent Ca^{2+} release from mitochondria (Epps et al., 1982), N-type Ca^{2+} channel activity (Mackie et al., 1993), and gap-junction conductance (Venance et al., 1995). Although the complete mechanisms of NAE function in mammalian cells remain somewhat obscure, the cannabinoid receptors CB1 and CB2 for anandamide (Devane et al., 1992) and palimitoylethanolamide (Facci et al., 1995), respectively, are a likely site of action. A recent analysis of plant cell perception of systemin (the endogenous wound-induced signal; Pearce et al., 1991) indicated that the N-terminal domain was essential for receptor binding but it inhibited medium alkalinization induced by native systemin (Meindi et al., 1998). While we do not yet have any direct evidence, it seems reasonable to speculate that plants have a receptor for NAE 14:0 (similar to the CB receptor in vertebrates) that activates a pathway to regulate the elicitor-induced changes in cellular metabolism.

Recently, a pathogen-induced NO-signaling pathway that compliments events mediated by H_2O_2 has been identified in plants (for review, see Camp et al., 1998). Activation of this NO pathway leads to activation of PAL expression and potentiation of H_2O_2-mediated cell death in Arabidopsis leaves (Delledonne et al., 1998). In animal cells, NO is a well-known molecular component of signal transduction pathways controlling an array of physiological functions including host responses to infection (for review, see Hausladen and Stamler, 1998). In addition, the CB receptor (for NAE) is coupled to NO release in both vertebrate and invertebrate cells (Stefano et al., 1998a). Exogenously supplied anandamide also triggered NO release in leech and mussel ganglia in a concentration-dependent manner (Stefano et al., 1998b). Therefore, it is possible that NAE 14:0 release may act through a NO-mediated pathway to activate PAL expression. Consequently, NAPE/NAE metabolism may represent a conserved mechanism in multicellular eukaryotic organisms for signaling pathogen invasion at the cellular level.

It should be emphasized that perception of pathogen elicitors by plant cells involves the coordinate action of several phospholipases (Chapman, 1998) which together most certainly produce a multitude of lipid-derived signaling molecules. Clearly, NAE release represents only part of a complex scheme of signaling circuits that provides plant cells the flexibility to respond to multiple environmental stresses. A complete understanding of defense signal transduction pathways must take into account other PLD products (such as phosphatidic acid) as well as products of phosphatidylinositol-specific phospholipase C and phospholipase A activities. As efforts continue to focus on the identification and quantification of new lipid mediators, the interaction of phospholipase-mediated signaling pathways leading to plant defense responses will become better understood.

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